Thesis submitted for the Degree

of Ph.D.,

in the University of Edinburgh.

A Study of the Lignin Fraction of Foods and its Significance in Animal Nutrition Studies.

by

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1951

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CHAPTER I

Introduction and Review of Literature

1.1 Introduction

The need for a more accurate and satisfactory scheme for the analysis of plant material is well realised at the present time (Norman, 1935, Crampton and Maynard, 1938, Ferguson, 1948, Smith, 1950) and particular attention is being paid to the structural constituents (mainly lignin and cellulose) of fibrous and leafy grassland products. The chemical and physical properties of these structural materials may have a profound effect on their utilisation as well as on the utilisation of other associated plant constituents. The total digestible nutrients and starch equivalent of grass are reduced as the lignin and cellulose increase with approaching maturity.

Lignin is the most resistant fraction of the plant and is considered to be indigestible (Crampton and Maynard, 1938, Ellis et al. 1946, Ferguson, 1942, Dietrich and Konig, 1871, Stutzer, 1875, Paloheimo, 1925, Rogozinski and Starzweska, 1926,7,8) or of only very low digestibility. (Konig and Becker, 1910, Hale et al.1940, Louw, 1941, Ferguson, 1942). Konig et.al.,(1906) claimed that the digestibility of roughage by sheep is related to its lignin content, the/

the greater the percentage of lignin, the less digestible being the plant material. Moreover the increase in nutritive value following the artificial delignification of straw (Watson, 1941, Ferguson, 1942, 43, Woodman and Evans, 1947a) supports the view that lignin reduces the digestibility and this fraction can in fact serve as a better index of food value than the crude fibre content (Lancaster, 1943, Armstrong et al, 1950). In this connection Watson (1949 p.11) states: "The lignin should be borne in mind because it is of little use to animals, and as it gradually envelops the plant tissues so they become of decreasing value to the animal."

The partition of the 'crude carbohydrate' into 'crude fibre' and N-free extractives by the time-honoured Henneberg system (1860) is very empirical and has been seriously criticised on both chemical and physiological grounds. The original investigators themselves realised that the crude fibre fraction contained varying percentages of Carbon, Hydrogen and Oxygen, and many other workers have demonstrated its compositional variability. (Heuser, 1921, Schultze, 1892, Williams and Olmsted, 1935, Norman, 1939, Bondi and Meyer, 1943, Ferguson, 1942, Armstrong et al.1950) This variability was found in the crude fibre of different plant species, and also in that of individual species/

species examined at different stages of growth, whilst in feeding experiments food fibre and faeces fibre were found to be of different composition. The unreliability of digestibility data based on this system of analysis is therefore obvious (Ferguson, 1942). Moreover the digestibility of crude fibre is often equal to or even greater than that of the nitrogen-free extractives (Morrison, 1936, Schneider, 1947.)

Armstrong et al. (1950) examined a random selection of the food analyses given in Morrison's (1940) tables and found that in 39% of the cases the digestibility of crude fibre was higher than that of nitrogen-free extractives. Thus the crude fibre can not be considered the least digestible fraction, and there is no valid physiological basis for such assumption. An examination of the digestibility data collected by Schneider (1947) shows that the digestibility of the crude fibre of Gramineae is generally equal to or greater than that of the nitrogen-free extractives whereas in the legumes the crude fibre is of lower digestibility than the nitrogen-free extractives.

Several attempts have been made to devise a more rational scheme of analysis (Remy, 1931, Crampton and Maynard, 1938, Williams and Olmsted, 1935, Davis and Miller/

Miller, 1939, Crampton and Whiting, 1943) in which lignin is determined as a separate fraction. Crampton and Maynard (1938) proposed a method for the partition of 'crude carbohydrate' into lignin, cellulose, and 'other carbohydrates', the latter being determined by difference and many other chemists have followed the same line using various methods for the determination of lignin and cellulose (Hale et al, 1940,1947, Lancaster, 1945, Ellis et al, 1946, Ferguson, 1942, Woodman and Evans, 1947a,b). Furthermore on the assumption that the lignin of the food is completely recovered in the faeces, Ellis et al (1946) suggested using lignin as a natural marker in a lignin ratio technique for computing the digestibilities of other food constituents. The validity of the method was confirmed by other workers (Swift et al, 1947, Forbes and Garriguss 1947, 1950a), and its use was extended to the determination of the dry matter consumption of grazing animals.

There are thus several advantages in the separate determination of lignin and as this fraction contains an aromatic nucleus (Phillips, 1946 p.345 Erdtman, 1950), and has a higher energy value than true carbohydrate (Rubner, 1928a) it is necessary that it should be clearly differentiated from the carbohydrates and not divided between the 'crude fibre' and 'nitrogen/

'nitrogen-free extractives' as in the conventional method (Norman, 1935, Bondi and Meyer, 1943, Ferguson, 1942, Armstrong et al, 1950). If there is some digestion of lignin, then its intermediary metabolism in the animal body is obviously important. The aromatic group is essential for the animal, which is unable to synthesise it, but at the same time the animal has only a limited tolerance of aromatic substances. Besides these nutritional considerations, lignin is an important factor in the decomposition of plant material in manures, composts and in the soil, leading to 'humin' and 'humic' acid formation. Its importance in forestry and wood utilisation needs no emphasis.

The study of lignin and its metabolism requires a reliable analytical method for the determination of this fraction in foodstuffs. Our knowledge of lignin chemistry is incomplete, however, and the methods of determination at present available have no reliable basis. Norman (1937,p.177) reviewing the methods of lignin determination commented, "the inaccuracies of older methods are not generally realized. Some of the published figures for lignin content are too high, particularly those carried out on materials with a high pentose or protein content. Many conclusions bearing on changes in lignin content, or digestibility and decomposability of lignin, are unsound or completely erroneous/

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erroneous for this reason." Later, Phillips (1946) stated in his review on lignin metabolism, "Some investigators claim and others deny that lignin is in part digested by certain animals. Moreover, many of the available data were obtained when the limitations of our analytical methods were not fully realised, and investigators did not, accordingly, adopt a sufficiently critical attitude in interpreting their results." The same author (1946) P 634 indicated clearly that until we know definitely what we are trying to determine, no rational method can possibly be developed, and we must be satisfied with approximate determinations only. The situation indeed is more complicated and doubtful. The range of lignin digestibility recorded in the literature is very wide, from -46.3% to +64% (Lancaster, 1943, Bondi and Meyer, 1943). Significant negative digestibility is biologically impossible as the animal cannot synthesize lignin and it is very unlikely that lignin can be synthesized in the digestive tract by the micro-organisms. This fact may indicate that the current methods of lignin determination are far from being even approximate, particularly when applied to young green plants. Although much work has been done in recent years to perfect the methods, the results indicate that the methods are still inadequate.

The/

The work to be reported in this thesis was conducted mainly to determine the reliability of the methods used in lignin determination and their suitability for application to animal nutrition studies. Despite the handicap of our present inadequate knowledge of lignin chemistry it was hoped that a systematic examination of the various processes commonly used in lignin determination and of the nature and purity of the product at each stage, would lead to a better understanding of the factors involved. In particular it was hoped to obtain some explanation of the divergent opinions and conflicting results that have been reported, with possibly an indication of the means to improve the techniques so as to obtain more reproducible results and a product of more constant composition. Alternatively, it was felt that the examination might reveal faulty assumptions or indicate the importance of previously unrecognised factors, necessitating the abandonment of some of the processes at present employed. It was regarded as of fundamental importance that this detailed investigation of the subject of lignin determination should be carried out before embarking on any large scale nutrition investigations or pasture studies requiring the determination of lignin.

In order to indicate the present position, and

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in particular the factors of debatable importance as well as those requiring investigation a review of the subject is presented. Particular attention is directed to the quantitative determination of lignin and its application in the evaluation of the nutritive values of foodstuffs, particularly grassland products which are of considerable economic importance and show great variations in composition. As a study of the analytical methods for lignin, necessitates a knowledge of the chemistry of this fraction a summary is included covering the main aspects related to lignin determination.

1.2. The Lignin Chemistry

The extensive literature on this subject has been reviewed by the following authors: Fuchs (1926), Norman (1937, p.151), Freudenberg (1939), Hibbert (1942), Percival and Fischer (1944), Dorée (1947, p. 484), Phillips (1946, p. 272), Erdtman (1950) and many others.

1.2.1. Terminology The name lignin was first introduced into scientific literature by de Candolle (1833), a plant physiologist, and the term lignification has been widely used to designate the thickening of cell walls by the deposition of lignin. Botanically lignin has been rather vaguely defined as

"A/

"A complicated mixture of substances formed by certain cells of plants and deposited in thickened cell walls, particularly in wood tissues", (Tweney and Hughes, 1947).

Payen (1838) used the name lignin rather more specifically, to describe the "true woody material", a substance or substances with a higher percentage of carbon than cellulose. Later he used it to designate the "incrusting materials", assuming that cellulose was surrounded or impregnated with it.

Schulze (1857) described as lignin the substances removed in the oxidation of wood to obtain cellulose, whilst Fremy (1859) defined "Ligneux" as the residual material after treating wood with 72% sulphuric acid. This is very near to the general sense in which the majority of chemists use the term, and in fact the plant material remaining after treatment with 72% sulphuric acid (or other similar acid treatment) is the basis of most current methods for lignin determination.

A precise chemical definition of lignin has not yet been achieved. It is probably a mixture of related compounds similar in structure, but differing in the nature of the side-chain groups, substituting groups or degree of polymerisation; it is characterized by its resistance to strong acid, its aromatic nature, and/

and the presence of a stable methoxyl group attached to the aromatic nucleus. The detailed structure and mode of linkage between the constituent units are not certain. (Norman, 1937, p.151, Phillips, 1946, p.345, Erdtman, 1950).

According to the method of isolation, there appear in the literature several lignins usually characterized by the name of the author or the reagent, such as - acid lignin (Klason Lignin by sulphuric acid, Willstatter Lignin by fuming hydrochloric acid) alkali lignin or metalignin, ester lignin (by acetoacetic ester) and many others. The term "native lignin", or usually Brauns lignin, refers to lignin isolated by very mild treatment, and according to Brauns it is unchanged lignin similar to that "in situ".

There is as yet no agreed terminology, particularly in quantitative determinations (usually using strong acids), and the lignin fraction may be given various names, e.g. lignin, apparent lignin, crude lignin, ash-free lignin, pure lignin, true lignin, corrected lignin. The system of nomenclature adopted in the quantitative work to be reported in this thesis, is given on page 50.

1.2.2. Identification The presence of lignified tissues/

tissues is usually recognised by colour tests most of which depend on the presence of aldehydic or phenolic groups. The reagents used may be inorganic but are more usually aromatic substances - phenols or bases. In the case of the phenols the plant material is moistened with dilute hydrochloric acid and then treated with an aqueous or alcoholic solution of the reagent. With basic compounds the reaction is carried out by adding a 1% solution of the base in hydrochloric or sulphuric acid. None of these tests appears to be specific for lignin and it is doubtful whether the colours given are due to specific groups in the lignin molecule or to the presence of impurities (Phillips, 1946, p 278). Since isolated "Willstatter lignin" and "phenol lignin" gave the colour reactions characteristic of lignin in the tissues, Phillips (1946, p.280) concluded that the reactions are really due to the lignin itself and not to minor associates, although he found that alkali lignin did not give the typical colour reactions. Nickel (1889) reported that wood treated with sodium bisulphite was no longer coloured by the aniline sulphate or phloroglucinol reagents and according to Loree (1947, p.336) the phloroglucinol reaction is not obtained after treating wood with small quantities of chlorine or hydroxylamine or after gentle oxidation, although the lignin is still present/

present and apparently unaltered. Russel (1948) found that alkali lignin and a synthetic lignin preparation both gave the characteristic violet-red colour reaction with phloroglucinol (in 12% hydrochloric acid) but Harlow (1927, 1928) found that the colour given by phloroglucinol varied considerably and this worker suggested that too great reliance on the test might lead to error.

Maüle, (1900) found that lignified material when left in a 1% potassium permanganate solution for 5 minutes, washed, treated with dilute hydrochloric acid, washed and then immersed in an ammonia solution, acquired a deep red colour. This is a fairly widely used test and according to Browne (1901) the reaction appears to be due to the deposition of manganese dioxide on the wood. This reacts with the hydrochloric acid to produce chlorine which forms, with the alkali, the red coloured compound. A similar mechanism was suggested by Crocker (1921). Although Dorée (1947, 538) regards the colour as probably due to impurities rather than lignin, Wise et al (1946) state that the Maüle test is so sensitive that even traces of lignin respond to it.

The chlorine-sodium sulphite test is a very sensitive one and probably the most reliable from the chemical standpoint (Dorée, 1947, p.339). When isolated/

isolated lignin or lignified material is treated with chlorine (or hypochlorite and dilute mineral acid) a yellow colour is produced and further treatment with dilute sodium sulphite gives a pink to purple-red colour. Campbell (1937) claimed that this reaction is probably specific for compounds containing the 1, 2, 3 trihydroxybenzene nucleus and in 1938 they reported the colour to be given by compounds having this nucleus modified by other substituents. The colour is produced from a secondary product of the chlorination, identified as a phenol, but it is not produced in alkaline solution when an excess of chlorine is present (Campbell and McCowan, 1939). Campbell (1937), found that the reaction is given by cold or hot aqueous extracts of wood, by the acid filtrate from lignin determinations and by cold aqueous extracts of isolated sulphuric acid lignin. Angiosperms (plants with covered-seeds) usually give a more intense red colour than gymnosperms (bare-seeded plants, e.g. soft woods) and green materials (Doree 1947, p.339, Norman 1937, p.182).

Moerke (1945) tried other amino compounds for detecting lignin (mainly in newsprint and pulps), and listed 25 compounds including tryptophane and 1, 2, 4, 5-tetrahydroxy - 3, 6 -diaminobenzene; the former gives a flesh-pink and the latter a maroon colour./

colour. He claimed that all these reagents gave similar colours with p-benzoquinone and concluded that lignin contains a quinone-like structure.

Doree (1947, p.339) recommends the identification of lignin by applying the chlorine-sodium sulphite test, both to the original material and to the residue isolated by treatment with 72% H_2SO_4 or fuming hydrochloric acid. He also recommends testing with ferric-ferricyanide which is probably a specific test depending upon reduction to the blue ferrous-ferricyanide by the reducing groups in lignin.

Folin's reagent (Folin and Denis, 1912) was used by Mehta, (1925b) for the quantitative determination of lignin extracted in alkaline solution, the reaction being with the phenolic group in lignin, for which the reagent is specific.

The ultraviolet absorption spectrum can also be used for lignin identification. Most of the work done in this field (Herzog and Himler, 1927, Hagglund and Klingstedt, 1931, Stamm et al, 1932, Patterson and Hibbert, 1943) indicates that lignin is aromatic in nature and has a characteristic absorption maximum in the region of 2800 Å. This band persists despite alteration of the lignin molecule by methylation, acetylation or treatment with phenol (Glading, 1940) and is shown by lignin isolated from different sources/

sources and by different methods. Although absorption spectrophotometry is useful, several other compounds e.g. tryptophane and tyrosine show the same absorption maximum (Sowden and De Long, 1949, Carpenter, 1948)

1.2.3 Physical Properties Lignin is an amorphous substance, decomposes without melting, and has a high refractive index (1.61), indicative of an aromatic structure (Freudenberg et al. 1929, 1931).

In addition to the characteristic absorption spectrum (I, 2.2) the X-ray pattern indicates the crystal lattice of an organic substance of high molecular weight, (Harris et al, 1937-1938) but on the other hand, the specific viscosity of certain lignins is not high (0.03 - 0.064) indicating that they are of relatively low molecular weight and not in the form of long chains (Staudinger and Dreher, 1936).

The minimum molecular weight recorded is 800 - 1000 although Bondi and Meyer (1948) obtained values as low as 604 using alkali lignin from young plants; the osmotic pressure and boiling point methods give values of 3900 ± 300, whilst diffusion methods give about 10,000 (Doree, 1947, p.486). Staudinger et al, (1936) showed that relatively simple structural units of lignin can be polymerised and depolymerised and that/

that colloidal properties develop with a comperatively small degree of polymerisation. Solutions of lignin are colloidal and show great capacity for dispersing insoluble substances such as barium sulphate. It is now recognised that lignin solutions may contain insoluble lignin, hemicellulose or even cellulose in colloidal suspension (Doree, 1947, p.485).

Rubner (1928a) found the heat of combustion of lignin isolated from wood to be 6.277 Cals. per g. of ash-free material.

Lignin "in situ" is little affected by organic solvents alone, but lignin preparations modified by the processes of isolation may pass into solution in certain solvents. From the plant tissues lignin passes into solution in alkalies more or less readily, according to circumstances, but complete extraction is not easily achieved. Lignin may dissolve partially in a solvent, due to some impurities or to the presence of molecules of relatively short chain length (low degree of polymerisation) which can be dissolved. This is perhaps a feature of many polymers.

Standnikov et al (1955) found that lignin possessed the property of adsorbing barium hydroxide to a constant degree and recommended its use for identification purposes.

1.2.4 Lignin Preparation Lignin is prepared from plant/

plant material either by the hydrolytic removal of associates leaving an insoluble lignin residue or the removal of lignin itself by means of suitable solvents. In all methods the finely ground plant material is first freed from extractives such as fatty materials, resins, organic acids, pigments and volatile oils. This is usually done by extraction with 1 : 2 ethanol-benzene solution. Additional preliminary treatments may also be used, e.g. extraction with cold or hot water, extraction with 5% sodium hydroxide solution, and hydrolysis with dilute mineral acids. It is generally believed that isolated lignin always undergoes some change, except in the case of Brauns 'native lignin' referred to previously. Alkali pretreatment has the disadvantage of removing some of the lignin.

1. 2.4.(i) Methods of hydrolysing the associates of lignin. Sulphuric acid (64 - 72%, (w/w) usually 72%) is commonly used to dissolve (or disperse) associates and concurrently to hydrolyse them. This method is usually associated with Klason's name although there are many modifications differing in acid concentration, and in time and temperature of contact. After dilution with water (usually to reduce the acid concentration to 3%), the mixture is hydrolysed, filtered, washed free from acid and dried. The final hydrolysis/

hydrolysis is intended to complete the hydrolysis of the carbohydrate.

Fuming hydrochloric acid (43-42%) may also be employed but its unpleasant nature limits its use. To obtain removal of carbohydrates the material should be finely ground, the concentration of the acid should be 72%, in the case of sulphuric or 43% with hydrochloric, and an excess of the acid should be used; the final hydrolysis should be for at least 2 hours. Paloheimo, (1928) using 70% sulphuric acid for lignin isolation stated that a long exposure to the acid caused 'humus' formation, whilst too short a treatment left some unhydrolysed carbohydrates. Sherrard and Harris (1932) pointed out that the temperature of the sulphuric acid should be carefully controlled, otherwise carbohydrate reversion products may contaminate the lignin isolated. These workers used 10 c.c. of 70% sulphuric acid per g. of wood with contact for 16 hours at 10°C, then diluted with water to 3% volumes hydrolysed under a reflux condenser for 4 hours, filtered and finally washed with hot water. Norman (1937 p.154) recommended a 5% sulphuric acid prehydrolysis to remove the polysaccharides which would form reversion products in the presence of the strong acid, particularly when the temperature is high or the contact time prolonged.

These/

These methods can, if the necessary precautions are taken, produce a reasonably pure product from wood but immature materials containing protein yield a product which contains nitrogen (Norman 1937, p.175) Modifications which may be made when these methods are applied to feeding stuffs will be considered later but the following points may be noted here:-

(a) Hagglund, (1923) and Hagglund and Bjorkman, (1924) stated that when fuming hydrochloric acid is allowed to react on lignified tissues, a portion of the lignin at first dissolves in the fuming acid, and later separates out.

(b) Schwabe and Ekenstarn, (1927) found that when fuming hydrochloric acid reacts on plant material the cellulose rapidly swells, preventing diffusion of the acid to the interior of the plant particles. The residual material may thus be contaminated with cellulose. They recommended starting with a low concentration (25%) of the acid, and increasing it gradually to 42-43%.

(c) Urban (1926) used a mixture of hydrochloric acid and phosphoric acid at 20°C for 6 hours to hydrolyse the polysaccharides then filtered without dilution, washing with the same mixture then successively with concentrated hydrochloric acid, dilute hydrochloric acid and hot water.

(d)/

(d) Schulbach et al (1952) used anhydrous hydrofluoric acid to remove lignin associates from wood by rapidly converting them into sugar anhydrides which are readily soluble in water. This method avoided the long contact needed for other acids.

(e) Kurschner (1940) working on spruce wood found that lignin isolated by various acid methods contained impurities such as cellulose, hemicellulose, and humus material from carbohydrate condensation. The impurities were determined by alcoholic nitration during which the lignin and much of the hemicellulose dissolved leaving a residue ranging from 0.28 to 43.5% A technical lignin treated similarly, gave only a trace of residue.

1.2.4.(ii) Methods of extracting lignin. (a) Alkali method: This widely-used method employs aqueous or alcoholic sodium hydroxide solution, followed by acidification. The ease of extraction depends on the character of the plant material; lignin fractions may be obtained from cereal straws by treatment with cold alcoholic or aqueous sodium hydroxide solutions, but for woods a more drastic treatment is required.

Beckman et al (1921) treated rye straw with 1.5% aqueous sodium hydroxide solution for 2 days at room temperature following which the filtrate from the pressed straw was acidified to give a concentration of/

of 2 - 2.5% free acid and then boiled for 5 - 10 minutes. The precipitate was filtered and washed with water. The same authors used alcoholic sodium hydroxide (20 g. sodium hydroxide in 400 c.c. water added to 600 c.c. 96% ethanol) for winter rye straw. After 2 days extraction the extract was neutralized with hydrochloric acid and the alcohol mostly distilled off under reduced pressure. The residual solution was acidified and the lignin filtered off and washed with water. The main object in adding the alcohol was to prevent the hemicellulose from dissolving in the alkaline medium.

Phillips (1928, 1930) and Phillips and Goss, (1936) used the alkali method for the fractional extraction of agricultural material such as corn cobs, oat hulls and oat straw. After extraction with ethenol-benzene the materials were treated successively with (1) 2% alcoholic sodium hydroxide solution, (2) 2% sodium hydroxide at 100°C, (3) 2% sodium hydroxide solution at 135°C and (4) 4% sodium hydroxide solution at 180°C.

Bondi and Meyer (1948) used 0.5N sodium hydroxide at 80 - 85°C to extract lignin from young grasses and legumes. The coarsely-ground dry material was extracted three times for periods of 8 hours each time then the extracts and washings were evaporated,

evaporated in vacuo to one third volume, cooled and acidified to precipitate lignin and hemicelluloses. The precipitate was redissolved in warm, 1.25 N sodium hydroxide, four volumes of alcohol (96%) added and the mixture made slightly acid. This reprecipitated the hemicellulose (and salts) but the lignin remained in solution. When most of the alcohol was driven off from the filtrate, the lignin separated out. It was purified by dissolving in warm 1.25 N sodium hydroxide then re-acidifying. High yields (4 - 9% of the dry material) are claimed for this method.

(b) Brauns' Method ("Native lignin") Brauns (1939, 1940) succeeded in extracting a lignin fraction from wood by using an organic solvent without the addition of any acid. Black spruce wood meal was extracted successively with cold water, ether and ethanol at room temperature and the alcoholic solution evaporated to dryness under reduced pressure. The residue, after washing with water and ether, was dissolved in dioxane and precipitated by pouring the solution into water. This process of solution in dioxane and precipitation with water was repeated until the methoxyl content remained constant. The residue was a light powder of creamy colour, and gave the typical purple colour reaction with phloroglucinol. The yield was 2 - 5% of the wood (approx. 9% of the

acid/

acid lignin).

Several workers have used the same technique with other soft woods (Brauns, 1945, Nord and Vitucci, 1948, Gottlieb and Geller, 1949), and recently Buchanan et al (1949) succeeded in isolating 'native lignin' from a hard wood. The main advantage of the method is the mild treatment which should not produce any changes in the lignin, but many workers believe that the fraction obtained cannot represent the lignin remaining in the wood.

(c) Lemmel's Method ("Ester" Lignin) Lemmel (1935) claimed that ethyl aceto-acetate in the presence of hydrochloric acid as catalyst can efficiently dissolve lignin from saw-dust at room temperature without chemical reaction or decomposition. The saw-dust is soaked in concentrated hydrochloric acid, then the ester is added. The lignin dissolves immediately, producing its characteristic dark brown colour. Lemmel also claimed that warming the mixture produces complete separation of the lignin, although no quantitative data have been given.

Virasoro, (1942) used this solvent with white quebracho wood, and found that the separation of lignin was not quantitative, although the greater part of it was isolated. He stated that a high hydrochloric acid concentration (10% of the amount of/

of aceto-acetic ester) and a temperature of 40°C for 24 hours gave the best extraction. The material was pretreated with ether, alcohol-benzene, and in some cases with water, before adding the solvent.

The same author (Virasoro, 1943) obtained 25.8% of lignin from the wood of Aspidosperma quebracho, Schlecht, and 14.6% from Salix humboltiano, Willd, by this method.

(d) Other common methods: Several other methods are available, in which the solvent usually reacts with lignin to form a derivative. They are used for technical purposes and/or chemical studies on lignin behaviour and structure.

The sulphite method is not generally used for the isolation of lignin, although it is widely employed in the wood industry. When the wood is heated under pressure with a solution of sulphurous acid and acid sulphites the lignin, together with many associates, goes into solution forming the so called "Lignin sulphonic acids". The mechanism of the reaction is still not completely understood (Erdtman, 1950).

Alcoholysis, produced by heating the lignified material with alcohol in the presence of hydrochloric acid, can remove some lignin. Other hydroxy compounds are also used, such as glycols, phenols, and other/

other alcohols, but the yield is always low and certain tautomeric substances like dioxane may be used. e.g. methanol, butyl and amyl alcohols, benzyl alcohol, ethylene glycol, and glycerol -monochlorohydrin. Ferric chloride may be used to replace the hydrochloric acid as catalyst. In all cases the alkyl or aryl group of the reacting hydroxy compound is found in combination with the lignin. The mechanism of the reaction is not clear (Phillips, 1946, p.228) Hagglund and Urban (1927, 1928) suggested that an acetal type of product is formed, but the evidence seems to be inconclusive. Another view is that a mixed ether may be produced.

Thioglycollic acid has also been used, forming a lignin derivative. According to Holmberg, (1936) the reaction is similar to that between a solution of bisulphite and sulphurous acid and lignin.

Formic acid lignin can be prepared by heating plant material with formic acid (92%) at 100°C in an atmosphere of nitrogen for 4½ days. The lignin is obtained by concentration of the extract and dilution with water (Phillips, 1946, p.295). The yield is high, but partial demethylation of the lignin occurs, (Borée, 1947, p.494).

Karrer and Widmer, (1921) found that wood, straw and other similar materials could be dissolved completely by/

by treatment for several hours with acetylbromide. A bromo derivative of lignin can be obtained from the precipitate which is formed when the solution is poured onto ice.

Glacial acetic acid has been used by Friedrich (1928) and Bell and Wright (1950) for lignin preparation. The latter authors isolated from yellow birch a lignin-carbohydrate complex and claimed that most processes of lignin preparation involved a hydrolysis of this complex into the isolated lignin and carbohydrates.

1. 2.5. Chemical Groups The presence of certain characteristic groups in the lignin molecule is responsible for the various changes that occur when it is isolated. There are always differences between the lignin residues obtained by different methods of extraction so that the question always arises: Is a certain group really typical of the original lignin in the plant or is it acquired during the course of preparation?

(a) Methoxyl group (OCH_3) The presence of this group has been definitely established in lignin prepared from different sources and by various methods. The stable ether linkage enables the group to resist several drastic methods of preparation, unlike the ester linkage, present in other plant fractions, (e.g. pectins)/

(e.g. pectins), which is easily de-esterified.

If no alcohols are used in the preparation of lignin, no alkoxy groups other than methoxyl are present: this has been definitely proved for spruce wood (Hagglund and Sundroos, 1924) and for straws and hulls (Phillips, 1946 p.295).

Freudenberg et al (1929) found that the methoxyl groups are attached to aromatic nuclei in the lignin molecule.

Methoxyl is the only group in lignin that may be easily determined. Its measurement provides almost the only means of assessing the purity of lignin and may also give quantitative information on changes brought about by additive reactions.

Recorded figures for the methoxyl content of lignin vary with the method of preparation, the plant material and the age of the plant. Lignin from mature tissues usually contains between 14 and 21%. Phillips and Goss, (1935) and Phillips et al (1939) found that in young barley and oats, the Willstatter lignin contains only 4.4% methoxyl, but the amount increases regularly as the plant grows older, the lignin from mature straw containing 16.5% methoxyl. Bondi and Meyer (1948) concluded from their results with alkali lignin that whereas the lignin from Gramineae contains about 10% methoxyl, that obtained from legumes/

legumes has only about half as much. Manning and de Long (1941) found very little methoxyl (0.35-1.66%) in acid lignin from vegetable materials such as turnip, asparagus and spring beans and concluded that this was due to the high nitrogen content (2.4 - 8.5%). MacDougal and de Long (1942) obtained similar results (0.406 - 5.53% methoxyl and 3.16 - 8.5% nitrogen in the lignin residue). In later work with oats of various ages (from 32 to 140 days) McDougal and de Long (1948c) obtained results similar to those obtained by Phillips et al, (1939) (3.80% methoxyl in the lignin from young plants, increasing to 15.44% in the lignin of old plants). McDougal and de Long however, did not correct for the nitrogen and ash in their lignin preparations as did Phillips et al(1939).

The methoxyl content of 'native lignin' from soft woods (spruce and white Scots pine) is much less variable, and may be considered to be practically constant. (14.7% Brauns (1940), 15.5% Brauns and Hibbert (1935), 15.3% Schubert and Nord (1950a). Buchanan et al (1949) recorded 19.5% methoxyl in 'native lignin' from hard woods.

When a derivative is formed by a reaction between lignin and the extractant, the percentage of methoxyl decreases or increases according to the nature of the group/ attached to the lignin. It decreases/

decreases when a phenol is used, and increases when an alcohol is used. In the latter case the total alkoxyl content will increase and the hydriodic acid used for methoxyl determination will react in a similar manner with any alkoxyl group attached to lignin, forming alkyl iodide, and so increasing the apparent methoxyl content. It is, however possible to separate methyl iodide from methyl iodide and so correct for any ethyoxyl groups in lignin.

(b) Hydroxyl Group

The presence of hydroxyl groups in lignin is indicated by the fact that lignin can be acetylated and alkylated. With diazo-methane, only a fraction of the hydroxyl groups can be methylated, but complete methylation can be produced by dimethyl sulphate in alkali. This led to the conclusion that some of the hydroxyl groups are aliphatic and others are phenolic or enolic in character, apparently there are no primary hydroxyl groups in lignin. Freudenberg et al. (1929b) indicated that the lignin isolated by their method did not contain any free phenolic groups.

(c) Other Groups

The presence of an acetyl group in all lignified material is not certain. Various lignin fractions when distilled with dilute sulphuric acid or digested in the cold with sodium hydroxide, yield acetic/

acetic acid or sodium acetate, whereas cellulose treated in the same way does not yield these products. This was taken as evidence of the presence of an acetyl group in lignin, but other workers have demonstrated that this group is associated with carbohydrate and is not a part of the lignin complex.

Reduction of Fehling's solution or the formation of condensation products with phenylhydrazine is not usually regarded as adequate evidence for the presence of a carbonyl group although Brauns (1939,1940) claimed to have succeeded in preparing a hydrazone from his 'native lignin', and his structural formula for 'native lignin' involves an equilibrium between enol and keto forms.

The carboxyl group is apparently not present in lignin obtained from wood (Phillips, 1946 p.500). Kukharenko, (1950) recorded the presence of only 0.080% carboxyl in oak lignin and 0.212% in a soft wood lignin.

Certain isolated lignins when distilled with dilute hydrochloric or sulphuric acid, yield some formaldehyde (0.5 - 4%) but it is not certain whether this can be accepted as evidence of the presence of a methylenedioxy group in lignin.

The evidence regarding the presence of an ethylenic bond in lignin is also incomplete and different/

different preparations behave differently towards the common reagents used for detecting and estimating unsaturation. Klason, (1920) assumed that an ethylenic bond is present in lignin and that the formation of a stable sulphonic acid with sulphurous acid and bisulphites, occurs as follows:-

$$-\overset{|}{C} = \overset{|}{C}- \quad + \quad H_2SO_3 \quad = -\overset{|}{\underset{H}{C}} - \overset{|}{\underset{SO_3H}{C}}-$$

The mechanism of sulphonation is, however, still not fully understood and Erdtman, (1950) states that it is mainly due to substitution of hydroxyl groups. It is not certain whether halogenation of lignins takes place by addition, or substitution or by both substitution and addition.

2.6 Lignin reactions Some of these reactions have already been mentioned and in this brief account particular attention is given to mineral acid hydrolysis, commonly employed in lignin determination procedures. The various aromatic compounds which have been isolated from lignin are shown in the Appendix (page 26-31).

Acylation (usually acetylation), alkylation (usually methylation) and halogenation have been used to study the qualities of different lignin preparations. The extent of these reactions varies according/

according to the source of the lignin, the method of isolating it and the method of performing the reaction. The percentages of acyl or alkoxy groups or halogen in the products obtained cannot be taken as characteristic of the lignin in question unless they are maxima (Russel, 1948). This is probably not the case with many of the figures recorded so that the wide range of values is understandable and comparisons can only be made with reservation. This may also apply in the case of nitrolignin, where the nitro nitrogen is also variable. The ease with which lignin is nitrated is taken by some workers to indicate a phenolic constitution.

Oxidation of lignin even under mild conditions produces simple degradation products, like formic and oxalic acids; this is due to complete disruption of the molecule. In a few instances it was possible to isolate aromatic substances like benzene -1,2,4,5-tetra-carboxylic acid.

From the phenolic fraction of an oil obtained by distillation with zinc dust it was possible to identify guaiacol (II), see appendix page 27 , and 1-n-propyl-3-methoxy-4-hydroxybenzene (XI). Anisic acid (IV) was also identified.

In the alkali hydrolysates of lignin sulphonic acids, vanillin (VI), acetovanillone (X) and guaiacol have/

have been identified in the case of soft woods, and vanillin, syringaldehyde (XV) and acetosyringone (XVI) in hard woods.

Alcoholysis of soft woods produces guaiacyl derivatives, but in the case of hard woods guaiacyl and syringyl derivatives are obtained. Alcoholysis produces the same derivatives as alkaline hydrolysis.

Fusion of lignin with alkalies yields, in nearly all cases, catechol (I), protocatechuic acid (V) oxalic acid and some simple aliphatic acids. When spruce lignin was first fused with alkali, the products were further methylated and finally oxidised giving degradation products amongst which veratric acid (VIII), isohempinic acid (XIX) and veratroyl-formic acid (XXII) were identified.

Sulphonation of lignin has been extensively studied but is still not completely understood. Whereas lignin "in situ" dissolves in bisulphite solution, lignins isolated by mineral acids or by organic solvents with mineral acid catalyst are insoluble in hot bisulphite. This indicates that they have undergone changes in structure during isolation and the changes apparently involve the hydroxyl group of the lignin.

In these degradative reactions lignin from straws and corn cobs behaves similarly to lignin from hard/

hard woods and both guaiacyl and syringyl derivatives are formed, whereas from soft woods only guaiacyl derivatives have been identified. Very few investigations of this sort appear to have been carried out with lignin from young plants although Bondi¢ and Meyer (1948) claimed to have isolated vanillin and p-hydroxy benzaldehyde in the constant ratio of 2 : 1 from alkali lignin by oxidation with nitrobenzene. Moreover, after fusion with potassium hydroxide they identified catechol and protocatechuic acid. Alcoholysis was also employed, but no derivative of the syringyl type was obtained.

Hachihama and Jodai, (1943) by catalytic dehydrogenation produced derivatives with a three-carbon aliphatic side chain from soft wood lignin, and both guaiacyl and syringyl derivatives (also with a three carbon side chain) from hard wood and grass lignins.

The dry distillation of lignin produces hydrocarbons and lignin tar, a product similar to coal tar. Isolation of eugenol (XXIII) from this product confirms the view that lignin contains the groupings of coniferyl alcohol.(XXIV).

1 2.6. (i) Acid Hydrolysis Heuser and Schmelz (1920), heated lignin at 150-160°C with 5% hydrochloric acid in a sealed tube for 3.5 hours and found that practically/

practically none of the methoxyl content was lost, but when they repeated the operation three times at 170-180°C complete demethoxylation occurred.

Hagglund (1923) prepared acid lignin from spruce wood and hydrolysed it with fresh portions of 3% hydrochloric acid until the acid extract no longer reduced Fehling's solution. As 33.7% of the lignin was lost and the acid extract contained 15.8% of sugar he concluded that carbohydrates form a part of the lignin molecule. This is doubtful, however, and other other workers believe the result to be due to incomplete hydrolysis of the carbohydrate fraction during isolation (Heuser, 1923). Acid lignins have been obtained entirely free from furfural-yielding substances (Bondi and Meyer, 1948, Phillips, 1927, Powell and Whittaker, 1925).

Hagglund and Bjorkman (1924) distilled Willstater lignin with 12% hydrochloric acid, and obtained a substance which was later identified as formaldehyde.

Aronovsky and Gortner (1930) found that if wood was heated with water under pressure a part of the lignin was rendered soluble in alcohol, although the quantity of lignin determined by 72% sulphuric acid remained practically constant. They postulated that depolymerization of lignin took place, or the linkage between lignin and carbohydrate was ruptured. Similar/

Similar results and postulations were published by Overbeck and Muller (1942).

Phillips (1938), treated Willstatter lignin from wheat straw alternately with fuming hydrochloric acid at 8 - 10°C for 24 hours and with boiling 5% hydrochloric acid for one hour. The amount of lignin lost decreased progressively with the successive treatments but the percentage of methoxyl in the residue remained constant, from which this author inferred that a degradation occurred in the lignin complex as a whole, rather than a removal of impurities.

Bondi and Meyer (1948) applied vigorous hydrolysis (refluxing with 5 N H_2SO_4 for 24 hours) to alkali lignin prepared from young plants and claimed that 10 - 15% of the lignin was removed although the absolute nitrogen content of the residue remained constant; they did not recover any nitrogen in the hydrolysate. On the other hand Thomas and Armstrong, (1950) using an alkali lignin prepared according to Bondi and Meyer's procedure, were able to remove 50% of the nitrogen by hydrolysis for 5 hours with 2.5 N hydrochloric acid at 15 lb pressure. The same authors hydrolysed acid lignin (prepared by the method of Norman and Jenkins (1934b) from Moorgrass (Molinia caerulea) and from faeces of sheep fed on artificially dried/

dried grass) under the same conditions and found that more than 50% of the nitrogen was removed. Tyrosine, leucine, valine and alanine were identified in the hydrolysate.

Harris and Mitchell (1939) and Cohen and Harris (1937), showed that pretreatment of wood with boiling dilute mineral acid or even boiling water dissolves lignin and consequently lowers the yield of acid lignin.

2.6.(ii) Biochemical reactions Waksman (1946) stated that when lignin is treated with alkali and exposed to the air for sometime, it is gradually oxidized and transformed into a dark coloured substance similar in properties to the typical "humic acids" with loss of methoxyl. Waksman and Smith (1934) indicated that in the aerobic decomposition of plant material in composts and soils, the lignin molecule is attacked as a whole and the methoxyl content of the residual lignin is not modified. Under conditions which lead to lignin accumulation, as in peat bogs, the methoxyl content is gradually reduced.

Autoxidative fixation of ammonia (in the presence of oxygen and aqubus ammonia)by lignin has been demonstrated by Matson and Koutler-Andersson(1943) and by Bennett, (1949). The former workers found that the

the nitrogen largely remains in the lignin fraction obtained by 72% sulphuric acid, and the nitrogen fixed also resists the action of strong alkali; they contended that simultaneous oxidation and nitrogen fixation took place by way of the phenolic hydroxy group. The latter worker, using a commercial lignin with 20.5% methoxyl found that the oxidised lignin contained 7.22% nitrogen and 17.2% methoxyl and when fully methylated contained 2.60% nitrogen and 29.2% methoxyl; thus a fraction of the fixed nitrogen resisted the somewhat drastic methylation procedure. Moreover the fully methylated lignin (33.0% methoxyl) fixed only 0.36% nitrogen. This methylation study seems to support Matson's view that the reaction occurs via the phenolic group.

Bailey (1948) digesting soft and hard woods with amino-butanol or nitro-butanol at various pH's for four hours at 160°C was able to isolate from the digest a lignin residue, containing 1.4 - 2.5% nitrogen. He concluded that condensation takes place between the lignin and the alcohol through the hydroxyl group of the alcohol.

Gottlieb and Geller (1949) using Brauns' lignin from Sprucewood as substrate for an enzyme preparation from commercial mushroom spawn, were able to demonstrate oxygen absorption by the Warburg manometric technique/

technique indicating enzyme activity. They showed that the enzyme is not similar to any of the known phenol oxidases (as tyrosinase). Nord and Vitucci (1948) have isolated p-methoxy cinnamate from the metabolites of Lentinus lepiduis (a wood rottening fungus) and attempts are being made to isolate specific fungi which will attack only lignin.

Cellulolytic enzymes obtained from snakes have been used to isolate lignin from woods (Fernandez and Regueiro, 1946). It was also demonstrated by Schubert and Nord (1950a) that after treatment of wood with the cellulose-attacking fungus that causes brown rot, it is possible to isolate almost twice as much Brauns native lignin as is obtained from untreated wood.

Other interesting enzymatic experiments with lignin have been reported by Freudenberg and Richtzenhain (1947) and Freudenberg (1949). The former workers (using the Barcoft-Warburg method and an enzyme preparation from mushroom) showed that several guaiacyl and syringyl derivatives can absorb oxygen and the absorption curves are affected by other groups in the derivatives. Freudenberg (1949) aerating coniferyl alcohol for 3 days at 20°C with 'dehydrase' from mushroom, claimed to obtain a dehydropolymerizate of the coniferyl alcohol in approximately/

approximately 90% yield. The products exhibited the characteristics of spruce native lignin such as colour reactions, response to methylation, sulphonation, Klason procedure for lignin determination and distillation etc. This author added that a polymer prepared from coniferyl alcohol had the same composition as the enzymatically prepared dehydropolymerizate, but differed in chemical and physical properties.

The digestion of lignin by the animal has been reported by several workers (Rubner, 1928b, Bondi and Meyer, 1943); the process is not bacterial in nature but appears to be enzymatic (Phillips, 1929, Hale et al, 1940).

It has been reported (Boruff and Buswell, 1934, Levine et al, 1935) that isolated lignin has a bacteriostatic action under anaerobic conditions. Levine et al (1935) suggested that the inhibitory effect is probably due to a protein precipitating action rather than to any specific toxic effect on the micro-organisms. Fuller and Norman (1943) believed that the effect of lignin in reducing the availability of plant cellulose to bacterial action, is mainly physical.

The validity of much of the biological work on lignin depends on the accuracy of the methods of determination/

determination used so that interpretations of many of the earlier experimental results must be taken with reservation.

1.2.7 Elementary Composition and Constitution The elementary composition of lignin varies somewhat with the method of isolation and with the source but the proportions of carbon, hydrogen and oxygen in various lignins are fairly constant. Most of the chemical work has been with wood, cereal straws and corn cobs, where the lignin is assumed to be practically free from nitrogen. The composition is 61.0 - 64.9% carbon, and 4.9 - 6.2% hydrogen, whereas a typical carbohydrate contains 44.3% carbon and 6.2% hydrogen. The difference in carbon content accounts for the higher calorific value of lignin.

There are many divergent opinions regarding the constitution of lignin, which has been variously claimed to belong to the aliphatic, aromatic, hydroaromatic, or heterocyclic series. Neither the theory that lignin is related structurally to carbohydrate (Willstätter and Kalb, 1922) nor the theory that it is an artefact from methylated carbohydrate, has received support. The methylated carbohydrate appears to be a purely hypothetical substance which has never been demonstrated (Phillips, 1946, p.345). The evidence of degradation products and spectrochemical/

chemical analysis indicates that lignin contains an aromatic nucleus.

Although many formulae have been given for lignin, they are all speculative in character and most of them have had to be modified from time to time to accord with new information. Some of these proposed formulae are given in the Appendix (page 26) and are referred to below.

Cross and Bevan (1905-1910) were the first to propose a constitutional formula (XXVII) but this is no longer in harmony with established lignin reactions.

Klason (1908, 1920a,b, 1922) suggested that lignin is essentially a polymer or condensation product of coniferyl alcohol (XXIV)(or coniferyl aldehyde (XXV)) and hydroxyconiferyl alcohol (XXVI). He was the first to suggest an aromatic structure and his theory is in harmony with the present opinion of several investigators who regard lignin as essentially a condensation polymer of simple units of the types represented by formulae XXIX and XXX.

Phillips (1931) and Kurschner (1925) suggested a benzenoid structure for lignin and the latter worker suggested a formula (XXXI) which contains an unsaturated bond and is a derivative of coniferyl alcohol. Lignin does not, however, behave like a true unsaturated compound as this formula would suggest/

suggest. When heated with hydriodic acid coniferyl alcohol gives both methyl and ethyl iodide (the latter resulting from a degradation of the propylene side chain), and the compound (XXXI) formulated by Kurschner should also yield ethyl iodide. When Spruce lignin is treated with hydriodic acid, however, no ethyliodide is formed so that Kurschner's formula must be presumed to be incorrect.

Freudenberg (1928, 1932) for several years maintained that lignin is a condensation product of the type indicated by formulae (XXIX) and (XXXII). He also believed that type (XXX) may be present and that the type XXXII occurs at the end of the chain. The linkage is of an ether type, between the primary hydroxyl group in the side chain of one unit and the phenolic hydroxy group of a second unit, forming a linear condensation product (XXXIII). This worker made several modifications later, and suggested that the side chain in the structural unit may be as in formulae (XXXIV), (XXXV) or (XXXVI). In the case of Spruce wood, the aromatic nuclei are only of the types (XXXVII) and (XXXVIII), but in hardwoods, type (XXXIX) is also present. The existence of a piperonyl group (containing the methylene dioxy group) in lignin is doubted by some workers.

Subsequently Freudenberg abandoned his belief in an/

an ether-type linkage and considered the union to be of a carbon-to-carbon type involving oxygen ring formation between the side chain and the aromatic nucleus as in formula (XL) .

Hibbert (1942) postulated that the ethanolysis degradation products of lignin such as α-hydroxy propiovanillone (XLII) and α-hydroxypropiosyringone (XLIII), vanilloylketones and syringoylmethylketones, may only represent stable end-products originating from dimeric compounds like dehydrodiisoeugenol (XLVI). These monomers are therefore not necessarily the true lignin building units. According to Hibbert the lignin progenitors in the plant are formed largely from a series of polymers of the dehydrodiisoeugenol type (XLVI) derived possibly from a monomer such as oxyconiferyl alcohol. This would not apply in the case of the syringyl derivatives where the two ortho-positions (relative to the phenol group) are blocked, so Hibbert suggested that the syringyl units exist as ethers in the woody tissues.

Erdtman (1950) has indicated that the belief that lignin is to a large extent built of units of types XLIV and XLV is in agreement with Holmberg and Freudenberg's analytical results which give an average composition of $[C_9H_9O_3.OCH_3]_n$.

Brauns' (1939, 1940) formula for the native lignin/

lignin from Spruce wood is claimed to be based on clear-cut analytical data and methylating reactions. The molecule contains four methoxyl and five hydroxyl groups one of which can show enolization (XLI) Earlier (1935) Brauns and Hibbert gave a slightly different formula containing five methoxyl groups instead of four. Buchanan, Brauns and Leaf (1949) preparing a purified native lignin from a hard wood, found that it exhibited structural differences from soft wood native lignin; one unit of hard wood lignin contained six hydroxyl groups of which one was phenolic.

Since lignin is found naturally in plants in appreciable amounts many workers believe that it is likely to be a polymer of simple units as is commonly the case with other plant constituents.

1.3. Determination of Lignin and Sources of Errors.

At present there is no satisfactory method for the determination of lignin particularly in foodstuffs and young plants. The present methods are largely derived from those used in wood analysis, but their application to immature plants, even after several modifications is unsatisfactory. In this review of the subject a very brief account of the older methods is presented, more detailed attention being paid to recent developments, particularly in relation to foodstuffs and young plants.

The methods of determination can be classified as:-

(1) Indirect methods, in which the lignin is obtained by difference or is based on the determination of a chemical group or colour development specific to lignin. These are seldom used nowadays.

(2) Direct methods in which the lignin is isolated, usually by hydrolysing associates with strong mineral acids.

1.3.1 Indirect Methods (a) Schulze (1857) oxidised lignified material with nitric acid and potassium chlorate, and assumed the loss in weight to be lignin. The results obtained are too high because other substances are oxidised e.g. hemicelluloses and perhaps/

perhaps some cellulose.

(b) The Holocellulose method. Hollocellulose determination is commonly applied to wood. Since holocellulose is considered to represent the whole carbohydrate fraction of wood its determination enables lignin to be determined by difference. Holocellulose is determined by the removal of lignin after chlorination usually with chlorine gas, followed by washing with alcoholic ethanolamine (Van Beckum and Ritter, 1937) or with a sodium hypochlorite-acetic acid mixture (Wise et al, 1946). Van Beckum and Ritter's method has been used by Manning and de Long (1941) for vegetable material. The results are usually high, particularly in the case of materials containing proteins since the amino groups are rapidly oxidised by the chlorine with the consequent breakdown of the molecule (Norman 1937, p.19).

(c) Bendikt and Bamberger (1890), determined lignin in wood on the basis of the methoxyl content assuming that lignin is the only substance in the plant containing methoxyl and that the methoxyl content of the lignin from various sources is the same. Neither of these assumptions is entirely correct. They may be approximately so for wood (Doree, 1947, p.368) but not for other plant material which contains carbohydrates with methoxyl groups (e.g. pectins) in appreciable/

appreciable amounts.

(d) Cross et al (1907) used the reaction of phloroglucinol with lignin in hydrochloric acid, and back-titrated the uncombined phloroglucinol with standard furfural or formaldehyde in hydrochloric acid solution. This procedure was found to give higher results than the 72% sulphuric acid or the fuming hydrochloric acid methods (Venkateswaren, 1925).

(e) Seidel (1907) measured the quantity of nitrogen oxides produced when lignin-containing material was treated with nitric acid, and calculated the lignin content using special factors.

(f) Mehta (1925, b) used a colorimetric method, extracting lignin with alkali under pressure, and using Folin's reagent, (a solution of phosphotungstic and phosphomolybdic acids in phosphoric acid; Folin and Denis, (1912)), to give a blue colour with the reactive phenolic groups in lignin. This method is limited to minute amounts of lignin and is not very accurate because degradation of lignin occurs under the conditions of extraction and in the presence of protein, tyrosine and tryptophane interfere.

(g) Crampton and Whiting (1943) proposed a new scheme for food-stuff analysis, in which ether extract, crude protein, ash, soluble carbohydrates and cellulose/

cellulose are determined and lignin obtained by difference. Crompton and Maynard's procedure (1936) was found to give higher results but the differences were variable.

(k) Other methods The methods based on the absorption of bromine (Kurschner and Wittenberger, 1939) or chlorine (Semmler and Pringsheim, 1919) by the lignin in plant material are of questionable value as the halogenation reactions of lignin are still not properly understood.

2.2 Direct Methods These methods depend on the insolubility of lignin in strong mixed acids which will dissolve associates after a period of reaction in the cold. Although a great deal of work has been carried out to elucidate the factors involved in these methods they are not yet fully known and several modifications in procedure appear to have been made without a full realization of the interacting factors involved and the sources of error.

The reagents commonly used to degrade and dissolve lignin associates are 72% (w/w) sulphuric acid and fuming hydrochloric acid (42 -43% w/w). Several mixtures of mineral acids have been used, the majority being mixtures of sulphuric acid and hydrochloric acid. Before the plant material is treated with these reagents, it is freed from fatty, waxy/

waxy and resinous materials by 'organic solvent extraction' (usually 1.: 2 alcohol-benzene mixture). This extraction may be followed by a 'pretreatment' involving boiling with water, dilute mineral acid hydrolysis, or enzymatic digestion and sometimes a combination of these pretreatments is used. After 'the main treatment' with strong mineral acid, there is usually a 'final treatment' of the residue with boiling dilute mineral acid. The final residue is washed free from acids, dried, weighed and reported as "lignin fraction" or "lignin residue", or it may be ashed to obtain 'ash-free lignin'. The methoxyl and nitrogen contents of the 'lignin fraction' or the 'ash-free lignin' may be determined and the 'ash-free lignin' may be corrected for crude protein by subtracting 6.25 times its nitrogen content, and reported as 'corrected lignin'. The terms appearing in inverted commas above are the terms used subsequently in this work.

The procedures employed in the various direct methods of determining lignin are summarised in Tables I & II in the Appendix, P. 19 and are discussed here under the following headings with particular reference to sources of error. (1) organic solvent extraction, (2) carbohydrate interference, (3) interference due to nitrogenous materials, (4) interference due/

due to ash, (5) other sources of error.

1.3.2.1 (i) Organic solvent extraction The presence of resinous and fatty substances in wood, has for a long time been known to increase the lignin fraction and to avoid this the generally accepted procedure is extraction with ethanol-benzene (1:2) first used by Konig and Rump (1914). With plant material this solvent mixture lowers the lignin yield more than the use of ether alone or of any other combination of solvents. (Norman, 1937 p.173). As there is evidence that some benzene may be retained in the plant material even after oven drying, a final wash with alcohol to remove the last traces of benzene is usually recommended. (Norman, 1937, p.173, Ellis et al. 1946).

Von Euler (1923) believed that the alcoholic extractives of wood belong to the lignin fraction and must be added to the final lignin residue. This view does not hold for plant material which contains many non-lignin constituents which are extractable by alcohol or ethanol-benzene, (e.g. pigments, crude fats and some monosaccharides). In quantity the ethanol-benzene extractives may be greater than the lignin itself. Thomas and Armstrong (1949) found that in some grasses this fraction reaches 25% of the dry matter, whereas the lignin does not exceed 10%.

10%.

Klason (1923) used hot 50% alcohol at first but later (1931) only ether. Ritter et al (1932) used extraction for 4 hours with ethanol-benzene, but recommended the omission of this operation with alkaline cooked pulp. Wise and Fairbrother (1951) found that extractions with hot water and ethanol-benzene have different effects on the final lignin yield, according to whether the plant material is hard wood or soft wood.

Since Brauns' native lignin is based on the alcohol extraction of wood this indicates the possibility that alcohol may dissolve some of the lignin from wood and this has been confirmed by other workers (Chastaignet, 1948, Von Euler, 1923). Chastaignet, (1948) found that although a fraction of wood lignin dissolves in ethanol-benzene this is less than when alcohol alone is used. Other workers found that with wood which has been cooked in water, a lignin fraction can be removed by alcohol at room temperature (Overbeck and Muller, 1942). Thus some pretreatments besides removing associates may render a part of the lignin soluble in the alcohol. In many methods pretreatment involving hydrolysis and/or enzymatic digestion is followed by washing the plant material with alcohol and ether then drying (Williams and Olmsted/

Olmsted, 1935, Crampton and Maynard, 1938, Davis and Miller, 1939, Ellis et al, 1946, Armitage et al.1948). The possible lignin-solvent effect of the alcohol in these cases seems to have been overlooked.

Ellis et al.,(1946) using various pretreatments with hay and oat clippings concluded that on the basis of final lignin yield 4 hours extraction with ethanol-benzene is as effective as 30 hours. This conclusion is not justified by their data, however, which indicate that 4 hours extraction with ethanol-benzene plus 1 hour hydrolysis with 5% sulphuric acid is nearly as efficient as 30 hours extraction plus 3 hours hydrolysis with 1% hydrochloric acid.

Armitage et al (1948) claimed that the omission from their procedure of the 24 hours solvent extraction produced higher lignin values (0.54%, 0.34% and 1.37% more lignin in the cases of clover, hay and grass respectively.)

Chastaignet, (1948) using ethanol-benzene (1:1) with wood, found no correlation between the state of division of the sample (shavings, raspings or sawdust) and the quantity of extractives obtained by Soxhlet extraction. He found that after 32 hours extraction some material is still being removed by the solvent, although for practical purposes adequate extraction is obtained in 8 hours. With the recommended/

recommended syphoning rate of 6 cycles per hour he found that some lignin is extracted by 95% ethanol, a smaller amount by ethanol-benzene (1:1) and almost none by benzene, acetone or methylene chloride. Roudier, (1948) published a standardized Soxhlet procedure for the extraction of wood with ethanol-benzene (1:1), and used 8 hours extraction at a rate of 4 cycles per hour.

Thomas and Armstrong (1949) using the procedure of Norman and Jenkins (1934b) for grasses, lucerne and faeces found that the yield of ethanol-benzene extractives was much higher after 30hours (15-31%) than after four hours (10-12%), but the yield of corrected lignin was a little lower in the former case than in the latter. The difference in lignin yields was probably significant and those workers preferred extraction for 30 hours. Phillips (1932) and Phillips and Goss (1936) also recommended 30 hours extraction and this is included in the official method of the A.O.A.C.

Simmons, (1940) used a 3 hours extraction with ethanol-benzene for wood pulps instead of the 48 hours extraction which had been used previously. He also omitted the hot water extraction and found no difference between 1:1 and 1:2 mixtures of alcohol-benzene

Thus there is no general agreement as to the conditions/

conditions of extraction. The times used vary between 2½ hours and 48 hours. It appears safer to use ethanol-benzene rather than alcohol alone. In wood the error due to removal of lignin is comparatively small as the extractives constitute about 3.5% of the wood whilst the lignin residue is about 25% or more; on the other hand in foodstuffs and young grasses, the error may be far greater as the lignin content is between 2 and 15% whereas the extractives may reach 31% (Thomas and Armstrong, 1949). This operation may be a source of error, either by dissolving some of the lignin, or by being inadequate, to remove associates which increase the lignin fraction.

1.3.2.2. Carbohydrate interference It has long been known that in the presence of strong acids certain sugars resinify or caramelise, producing humin-like insoluble products. The amount produced increases with the time of contact and with a rise in temperature. Palcheimo (1928) using 72% sulphuric acid, stated that long exposure of plant material to the acid causes humus formation whilst short treatment leaves unhydrolysed carbohydrates. The same author (1929) called attention to the fact that fructose and sucrose form insoluble products when subjected to strong acid treatment as in lignin determination. Norman and Jenkins (1934b) showed that xylose and arabinose/

arabinose, or their polysaccharides, as well as sucrose and fructose, when treated for 16 hours with 72% sulphuric acid followed by dilution (58 times) and boiling, produce resistant insoluble materials. Norman (1937 p.169) stated that there seemed to be a distinct difference between the behaviour of pentoses alone with acid, and their behaviour with acid in the presence of lignin. In the latter case the increase in lignin is far in excess of the insoluble material found in the absence of lignin. It was concluded that a reaction takes place between the sugars and lignin. In strong acids lignin was found to condense with phenols, aldehydes and ketones forming stable compounds. The pentoses alone in strong acids slowly form furfuraldehyde. When lignin in plant material is treated with strong acids the removal of associates sets free reactive groups in the lignin which will condense with aldehydes formed from pentoses. Even if lignin has already combined with formaldehyde it can still react with a phenol (Dorée, 1947, p.371). Moreover Norman (1937) p 171 has shown that although any sugar or other substance does not produce insoluble products with strong acid under the conditions used in lignin determination this is not conclusive evidence that it does not produce interference by condensation when lignin is present/

present. This fact has been overlooked by several workers.

Attempts have been made to remove interfering substances as far as possible by pretreatment prior to strong acid digestion and to modify the conditions of strong acid treatment in order to minimise the formation of humin products from associates. Ritter et al (1932) considered that 2 hours contact with 72% sulphuric acid at 20°C is sufficient to effect complete dissolution of the carbohydrates, but their published data show that a minimum lignin yield is obtained by using 72% sulphuric acid for 7 hours at 10°C.

Sanford and Campbell (1936) recommended 5 hours contact for hardwoods and 6 hours for soft woods at 10°C (using 72% sulphuric acid) but Norman (1937) [p 171] stated that the differences between 7 and 5 hours contact seemed to be within experimental error.

Norman and Jenkins (1934b) suggested a pretreatment with boiling 5% sulphuric acid for 1 hour. After boiling wood for 2 hours with 3% sulphuric acid Cohen and Harris (1937) found a humin-like substance in the filtrate; this was isolated and gave lignin reactions. They therefore recommended that all hydrolytic pretreatment, even with boiling water, should be omitted. In 1939, Harris and Mitchell, confirmed/

confirmed their findings. When maple wood was given 4 hours hydrolysis with 5% sulphuric acid, 22.5% of the lignin fraction was lost, but when hydrolysis was continued for 6 hours, the loss of lignin was slightly less; they concluded that the soluble lignin in the hydrolysate was converted again into an insoluble compound. In the case of Spruce wood hydrolysed with dilute mineral acids, they isolated from the filtrate a precipitate which dissolved in glacial acetic acid, alcohol and chloroform. When the glacial acetic acid solution was diluted with water they obtained a precipitate with the properties of lignin and containing 16.6% methoxyl. Their results also indicated that when heated on a waterbath lignin is more soluble in 2% hydrochloric acid than in 15% sulphuric acid. Using 2% hydrochloric acid pretreatment they obtained from maple wood a lignin fraction of 21.2% containing 20.5% methoxyl (4.35% absolute methoxyl) whereas using 15% sulphuric acid they obtained 19.4% lignin containing 20.3% methoxyl (3.94% absolute methoxyl). Similar results were observed after heating with mineral acids under pressure; using 1% sulphuric acid, at 120 lb pressure, 21.1% lignin was obtained containing 20.6% methoxyl (4.35% absolute methoxyl), whereas 7% sulphuric acid gave 25.4% lignin containing 18.4%/

18.4% methoxyl (4.67% absolute methoxyl). In the latter case some of the carbohydrate was converted into a lignin-like residue which increased the isolated lignin fraction and lowered its methoxyl percentage, although the lignin fraction contained more absolute lignin as judged by its absolute methoxyl content.

McDougal and De Long (1948a) using immature plants, found that pretreatment with dilute mineral acid lowered the absolute methoxyl content of the lignin fraction and they offered three possible explanations: (a) solution of some lignin during pretreatment, (b) demethoxylation of lignin during pretreatment, (c) removal of methoxyl-containing carbohydrates during pre-treatment. The first and third possibilities may both occur together but the second possibility seems to be improbable since Heuser and Shmelz (1920), using more drastic conditions found practically no loss in lignin methoxyl. It may be argued, however, that the effect of acid hydrolysis on isolated lignin may not be analogous to the effect on lignin in situ.

Norman (1937c) found that successive short-time hydrolysis with 5% sulphuric acid gave a lower yield of ash-free lignin from straw and oak wood, than a single continuous hydrolysis. McDougal and De Long (1948c)/

(1946c) using Manning and De Long's (1941) main and final treatment compared the effect of two pre-treatments on the lignin residue obtained from immature oats. The fresh material was subjected to two half-hour extractions with ether-water, followed by 3 hours extraction with fresh boiling 1% hydrochloric acid in a special apparatus, and finally 30 hours Soxhlet extraction with ethanol-benzene. This was compared with a similar treatment but using ordinary refluxing for 3 hours in the acid hydrolysis. They found that fresh acid hydrolysis had little effect on the absolute ash-free lignin, but reduced the methoxyl content of the lignin fraction from 4.5% to 2.75% and the nitrogen content from 2.70% to 1.98%. Although the ash-free lignin obtained by the two methods was similar (2.9%), their findings cannot be regarded as contrary to Norman's since they used a prolonged ethanol-benzene extraction which may overshadow the effect of fresh acid hydrolysis.

Phillips and Goss (1936b) disagreed with Cohen and Harris (1937) and Harris and Mitchell (1939) and considered that lignin results are of no value unless complete extraction with ethanol-benzene (30 hours), with hot water and with boiling 1% hydrochloric acid is employed. This is the basis of the organic/

organic solvent extraction and pretreatment used in the A.O.A.C. standard method. Later Phillips (1946, p.640) considered that in the case of lignified material, particularly wood, (other than young shoots) extraction with hot water and dilute mineral acids is not necessary.

Phillips and Goss (1938) found that in the presence of fuming hydrochloric acid several carbohydrates, either alone or with lignified material, did not produce any humin-like precipitate or increase the lignin yield. Only fructose or carbohydrates yielding fructose (e.g. sucrose, fructosans, fructosides) appeared to cause interference, and these are not naturally found in great quantity in plants. Norman and Jenkins' (1934a) results with 72% sulphuric acid, however, indicate that pentoses and pentosans produce more interference.

Hilpert and Littman (1934) treated carbohydrate materials with 72% sulphuric acid at 20 - 22°C or with fuming hydrochloric acid at 6°C for 48 hours, and obtained high yields of humin-like insoluble residues, but these conditions are not usually employed in lignin determination. Later (1935) using 72% sulphuric acid they found that the yield of the humin-like substance is greatly reduced by lowering the temperature. Other workers (Bamford and/

Table 1 Effect of Time and Temperature of 72% Sulphuric acid Treatment

(Manning and De Long, 1941)

Material	Treatment	% Ash-free Lignin (in oven dry tissue)	% Methoxyl (in Lignin Fraction)	% N (in lignin fraction)
Oat straw	a (2hrs at 10°C(a)	10.90 ± .31 (8)*	13.33	1.54
	b (16hrs at 10°C(b)	11.33 ± 0.45 (3)	-	-
	c (2hrs at 20°C(c)	11.72 ± 0.14 (8)	-	0.73
Turnip A	a	22.37 ± 0.22 (6)	0.35	2.41
	b	20.24 ± 0.47 (4)	0.70	4.29
String beans	a	12.65 ± .61 (4)	-	7.54
	b	7.31 ± 0.54 (4)	0.84	6.48
	c	9.55 ± 0.30 (3)	-	6.56
Beets A	a	12.21 ± 0.09 (4)	-	4.97
	b	7.38 ± 0.11 (3)	-	6.00
	c	9.68 ± 0.12 (3)	1.68	6.02
Asparagus B	a	20.20 ± 0.39 (5)	1.10	5.78
	b	14.59 ± 0.25	1.66	4.00

* Number of determinations shown in brackets.

and Campbell, 1936) found that several carbohydrates produced little or no insoluble residue with 72% sulphuric acid.

Manning and De Long (1941) studied the effect of time and temperature in the main treatment of vegetable materials and oat straw (using the A.O.A.C. pretreatment) with 72% sulphuric acid and employed (a) 2 hours at 10°C, (b) 16 hours at 10°C and (c) 2 hours at 20°C. They found (See table 1 above) that in 6 out of 10 samples treatment (b) gave a markedly lower ash-free lignin than the treatments (a); with the remaining samples there was no significant difference. The shorter time of contact produced a more contaminated lignin-residue as in 5 out of 7 cases the methoxyl percentage was lower. Increasing the time of contact raised the nitrogen percentage in 6 out of 9 cases. In 3 cases treatment (b) gave a lower ash-free lignin than treatment (c) and in the other cases there was no difference. The authors suggested that the high yield following the shorter periods of contact is due possibly to incomplete dispersion of the carbohydrate fraction; they preferred treatment (b) for lignin investigations with vegetable tissues. The work of these authors shows clearly that different plant materials may behave differently towards the same treatments, but the results are not absolutely/

absolutely conclusive and clearly show the danger of relying on the results from only a limited number of different materials. Their conclusions based on the percentage of methoxyl in the lignin fraction are defective, because the ash content of the lignin varies considerably even in one plant material and is of a high magnitude (McDougal and De Long, 1942). The same authors compared the use of 5% sulphuric acid and 1% hydrochloric acid in the pretreatment (after ethanol-benzene and hot water extraction) and found that the differences in yield and quality of ash free lignin were not consistent.

Phillips and Goss (1936[a]) using their method with straw, compared the effect of time of contact with fuming hydrochloric acid at 8 - 10°C). Contact for 2 to 6 hours gave a very high yield of ash-free lignin (31.53 - 28.91%), but after 18 hours contact the yield was much less (11.94%) and was practically unaffected by further prolonging the contact period to 48 hours. Examination of their data shows that the absolute ash and nitrogen contents of the lignin residue remained practically constant in all cases (ash 1.08%, and nitrogen 0.078% of the original dry matter). It seems that the contaminant after a short contact period is itself ash-free and nitrogen free; Most probably it is cellulose or oligosaccharide reprecipitated/

reprecipited when dilution of the hydrochloric acid occurs. This possibility of contaminating the lignin residue with reprecipitated carbohydrates on dilution of the strong acid seems to have been overlooked by most investigators although some workers using hydrochloric acid appears to have filtered off the lignin residue directly from the strong acid. (Schwalbe and Becker, 1919; Popoff, 1938).

Freudenberg and Dloetz (1940) have mentioned that although Klason worked out the sulphuric acid method there still remains the question of the best concentration. In the case of Spruce wood, the use of 66-75% sulphuric acid gives relatively uniform results; with more dilute acid, carbohydrates are not hydrolysed and with higher concentrations humin formation occurs; in both cases the yield of lignin increases. Klason lignin was defined as a substance characterized by insolubility in sulphuric acid at all concentrations, and determined with the acid concentration which gives a minimum value, when impurities are presumed to be absent. However this definition, they found, failed completely when Linden wood was used as no true minimum value was obtained. They indicated that the second most characteristic property of lignin is its methoxyl content which depends on the presence or absence of such impurities.

In/

In all cases when different acid concentrations are used in lignin determination the methoxyl content passes through a maximum and the authors proposed to determine the "pure" lignin having maximum methoxyl content. They gave a procedure based on Klason's but using different acid concentrations; some woods required 75% sulphuric acid whereas others required 66.4%.

Muller (1959), found that Di-fructose forms humin with 72% sulphuric acid and 38% hydrochloric acid and this humin is similar in composition to the lignin obtained from Spruce and Beech wood. By analysis of chlorolignins and chlorohumins, he found that the chief difference is the presence of alkyl groups in the chlorolignins. This worker has also pretreated straw with water and dilute hydrochloric acid for long and successive periods at boiling point (1, 4 and 7 hours with water followed by 4, 4 and 4 hours with 1% hydrochloric acid and finally 4 hours with water). He found a loss of 8.3% of lignin, and the residue obtained was not free from humin. This important observation indicates that even after prolonged hydrolysis, exceeding any commonly used pretreatment, humin formation still persists. Although this worker defined humin as 'the condensation products of carbohydrate obtained by acids' he did not give the analysis/

analysis of the 'humin' be obtained from straw. Ducet (1946), determined lignin in manure, after extraction with ethanol-benzene, 2% hydrochloric acid (to remove calcium compounds) and then with 2% ammonia (to remove humic acids). The pretreated residue was treated with 72% sulphuric acid, diluted 10 times and finally refluxed for 5 hours, the ash-free residue being recorded as (lignin + humin).

Although the insolubility of lignin in mineral acids is the basis of its determination, there are a few recorded cases indicating its solubility. Wiechert (1939) stated that although anhydrous hydrofluoric acid at -65°C can be used to determine lignin, at -10°C, the greater part of the lignin goes into solution along with mono-saccharides. Hilpert and Littman (1935) claimed that straw almost completely dissolves in 72% sulphuric acid at temperatures below -10°C.

The final treatment commonly used in lignin determination is dilution of the strong acid to a suitable volume with water followed by a period of boiling to complete the removal of contaminating carbohydrate. The boiling may be for a fixed time (usually 2 hours), but in some cases fresh dilute acid is used, repeatedly until the hydrolysates are free from reducing sugars. Phillips and Goss (1936) using their/

their A.O.A.C. procedure with straw, studied the effect of the time of the final hydrolysis on lignin yield, and concluded that 1 hour is the optimum as longer periods have negligible effect. (1, 2, 4, 6, 8 and 10 hours refluxing gave 11.67, 11.55, 11.45, 11.34, 11.30 and 11.39% of ash-free lignin, respectively.) They got similar results even after omitting the whole pretreatment from their procedure but in this case the lignin yield was significantly higher. It was concluded that the increase in the yield when pretreatment was omitted was due to contamination of the lignin with carbohydrates interacting with the strong acid and lignin. These carbohydrate contaminants are equally as resistant to the final hydrolysis as lignin itself. This latter conclusion may be incorrect however, and it is equally possible that the lignin "in situ" may be partially removed by the pretreatment, whereas the modified isolated lignin may be more resistant to the final hydrolysis. This explanation is more in harmony with the work of Cohen and others mentioned previously.

Although Norman recommended dilution of the sulphuric acid to 3% (Approx. 0.6N) Phillips and Goss employed dilution of fuming hydrochloric acid to about 5% (Approx 1.4N). Armitage et al (1948) compared 3% and 4.6% sulphuric acid in the final hydrolysis/

hydrolysis with young grass and straw and found the difference in the lignin obtained to be so small as to justify the use of 4.6% concentration in order to reduce the final volume. Ellis et al (1946) preferred to dilute the strong acid (72%) about 7 times, then filter and use fresh 3% sulphuric acid. This obviates the filtration of a large volume, and allows the final refluxing of the lignin residue to be in acid free from hydrolysed associates.

1.3.2.3 Interference due to Nitrogenous Materials

The majority of chemists regard the presence of nitrogen in isolated lignin as a sign of contamination with nitrogenous material. Paloheimo (1925, 1926, 1929) called attention to the fact that proteins are not completely hydrolysed by the strong mineral acid used in lignin determination and suggested that correction be made by subtracting 6.25 times the nitrogen content of the lignin. This correction has been used by many workers (Phillips, 1932, Armitage et al, 1948; Common, 1945; Rutledge and Common, 1947, 1948; Thomas and Armstrong, 1949, Armstrong et al 1950, Forbes and Carrigus, 1950b).

Attempts have also been made to minimise the contamination with nitrogen compounds as well as the elimination of carbohydrate interference. Ross and Hill (1929) considered it possible to block some reactive/

reactive grouping in lignin by reaction with formaldehyde and so prevent protein retention by the lignin; they then applied a correction for the formaldehyde used. Norman (1937, p.175) questioned this correction, stating that it is not of general application but varies according to the source of the lignin. Crampton and Maynard (1938) adopted the Ross-Hill device in a procedure which was later abandoned as unsatisfactory (Ellis et al.1946).

Norman and Jenkins (1934b) investigated the effect of protein contamination on lignin yield, and found that the increase in lignin cannot be predicted from the amount of nitrogen in it. They stated that the problem was very complex and that possibly an interaction between proteins and pentose constituents occurred. Norman (1937) studied the effect of adding proteins and amino acids to pre-treated wheat straw in the presence of 72% sulphuric acid and found that amino acids did not produce appreciable increases in lignin, although proteins did. He suggested that the nitrogen combined with lignin may not be in the form of protein, but probably as protein degradation products. Thomas and Armstrong (1949) by hydrolysis of acid lignin with 2.5 N hydrochloric acid for 5 hours at 15 lb. pressure were able to isolate several amino acids, so they concluded that

that at least some of the nitrogenous material in lignin is protein or degradation product of protein. They did not consider it likely that the nitrogen of lignin was in the form of free amino acids and suggested the use of the crude protein correction (N × 6.25) until further information is available. By the same vigorous hydrolysis they were able to remove about 50% of the nitrogen from a sample of alkali lignin prepared according to Bondi and Meyer's procedure (1948). The results of Thomas and Armstrong (1949) agree to some extent with Norman and Jenkins' but differ entirely from those of Bondi and Meyer (1948) who claimed that nitrogen is bound in the lignin molecule, because they found that pretreatment of young plant material with pepsin and hydrochloric acid did not reduce the nitrogen content of the isolated alkali lignin. Bondi and Meyer were also unable to remove nitrogenous material from alkali lignin by acid hydrolysis, or by distillation with concentrated caustic soda or treatment with sodium nitrite, this strengthens their view that the nitrogen is bound in the lignin molecule.

According to Norman (1937) the increase in lignin due to protein interference seems to be inversely proportional to the time of contact with 72% H_2SO_4 and is not reduced at a lower temperature. Manning/

Manning and De Long (1941), however, found that nitrogen interference increases with time. The usual procedures for avoiding carbohydrate interference, viz control of contact time and temperature, or pretreatment with 5% acid, do not reduce the nitrogen in the lignin itself, although 5% acid pretreatment reduces the nitrogen content of the plant material. Later Phillips (1939) studied the effects of several proteins alone or with pretreated plant material, during digestion with fuming hydrochloric acid. He confirmed Norman's finding that the calculated correction factor for the nitrogenous material in lignin varies between 2.58 and 12.4, thus indicating that Paloheimo's suggestion is not applicable in every case. About 56.5% of the nitrogen in the pretreated straw was recovered in the lignin, but only 9 to 24% of the added protein was recovered so Phillips concluded that added proteins are much more susceptible to the hydrolytic action of the strong acid than the nitrogen compounds in the straw: the latter must be considered as being quite different from proteins.

Remy (1931) and Williams and Olmsted (1935) used enzymatic pretreatment to remove protein materials before lignin determination, but did not examine the qualities of the lignin. Crampton and Maynard/

Maynard (1938) modified the Williams and Olmsted pretreatment by using pepsin and hydrochloric acid instead of the alkaline enzymatic medium which may dissolve a part of the lignin. Davis and Miller (1939) used an elaborate combined chemical and enzymatic method to remove carbohydrates and proteins before lignin isolation with 72% sulphuric acid but did not determine the nitrogen content of the lignin. They also used a contact time of 45 - 60 minutes, the adequacy of which is questionable.

Lancaster (1943) using Crampton and Maynard's procedure in digestion trials with sheep, found that discrepancies in lignin digestibility were due to protein interference. The nitrogen content of faecal lignin differed widely from that of food lignin; in the case of rape it was only 0.42% in the plant, but 4.51% in the faecal lignin. The main treatment in this procedure is open to criticism, but a similar finding with grasses was recently reported by Forbes and Garrigus (1950b) using the method of Ellis et al (1946). They found that the nitrogen percentage in faecal lignin in all but one case was higher than that in the lignin from the forage. When these authors corrected for lignin nitrogen some improvement was observed in the digestibility data.

Manning and De Long (1941) compared their procedure/

procedure with that of Crampton and Maynard (1938), and concluded that the long tedious peptic digestion had no advantage. They found that the percentage of nitrogen in the lignin fraction did not give a reliable indication of the effect of nitrogenous material on the lignin yield.

Ellis et al (1946) proposed a pretreatment with pepsin-hydrochloric acid followed by 5% sulphuric acid hydrolysis for 1 hour, but they based their conclusions entirely on the figures for ash-free lignin.

McDougal and De Long (1948a) using fresh immature grass, claimed that continuous extraction with boiling 1% hydrochloric acid in an apparatus designed to ensure contact of the material with only the pure reagent removes more nitrogen than the usual refluxing with dilute hydrochloric acid. These authors tried protein extractants as an alternative method of removing nitrogenous material from fresh plants. Ether-saturated water proved to be the best extractant and could remove 90 to 95% of the nitrogen from fresh young tissues. In the case of air dried material or old plants, however, it was found necessary to treat with hot 1% hydrochloric acid to remove similar amounts of nitrogen. They deduced that increasing age apparently brings about changes in protein/

protein solubility which are similar to those caused by drying young green material. They compared their proposed pretreatment (3 extractions in a Waring Blendor with ether-saturated water + 3 hours refluxing with 1% hydrochloric acid + 30 hours ethanol-benzene extraction) with the A.O.A.C. pretreatment (30 hours extraction with ethanol-benzene + 3 hours refluxing with water + 3 hours refluxing with 1% hydrochloric acid) using Manning and De Long's 72% sulphuric acid digestion and final treatment. The ash-free lignin from the former pretreatments was lower and contained less nitrogen, although the nitrogen content was still high (0.48 - 4.71%).

McDougal and De Long (1948b) tried to maintain reducing conditions during pretreatment by the use of hydrogen sulphide. They found that, judged by the nitrogen and methoxyl contents, this had no effect on the lignin fraction in fresh material. In the case of air-dried material, these conditions apparently reduced in a large measure the usual interference of nitrogen-containing substances. There is no clear explanation of this result, which may perhaps have been accidental.

Armitage et al (1948) tried several combinations of enzymatic and chemical pretreatments with young clover, and proposed pretreatment with 5% hydrochloric acid/

acid followed by trypsin-sodium carbonate digestion. Out of 13 combinations tested this was found to be the most practicable pretreatment to remove nitrogenous material from clover. They found that the effect on the yield of corrected lignin due to the 0.25% sodium carbonate solution used in the trypsin digestion was negligible. The yield of corrected lignin by their procedure was appreciably lower than that obtained by the Norman-Jenkins or Crampton and Maynard procedures and their lignin contained much less nitrogen than the others. Although these authors consider that even with their own method the nitrogen content is appreciable and believe that the figure for corrected lignin would be nearer to the true value, the lower lignin yield may not be entirely due to the removal of nitrogenous material because in their pretreatment they hydrolysed with 5% hydrochloric acid which is more effective than 5% sulphuric acid (used in the Norman-Jenkins procedure) in removing some of the lignin itself. Thomas and Armstrong (1949) compared the Norman-Jenkins (1934b) procedure with that of Ellis et al (1946), and found that after protein correction the results from both procedures were very similar: they therefore recommended the Norman-Jenkins method as being more practicable. The crude protein content of the ash-free/

ash-free lignin was between 12.92 and 44.26% in both cases (varying with the material examined). It is important to note that the results of Armitage et al (1948) do not support Norman and Jenkins' method, whereas those of Thomas and Armstrong do. This is because a suitable standard of comparison is really lacking as these workers relied only on the nitrogen content of the lignin which very probably does not give a good indication of the true lignin content of the isolated fraction although Thomas and Armstrong's investigations involving vigorous acid hydrolysis and identification of aminoacids in the hydrolysate strengthen the view that the nitrogenous material in the lignin fraction is probably of crude protein nature. There is reason to believe that even after allowing for the nitrogen, the corrected lignin may contain other contaminants. Some workers, using two different methods of determination have reported similar yields of ash-free lignin although the nitrogen and methoxyl contents of the lignin fractions may differ considerably. McDougal and De Long (1948a) employed a continous pretreatment with fresh 1% hydrochloric acid in their special apparatus and obtained 2.90% of ash-free lignin, containing 2.75% methoxyl and 1.98% nitrogen, but using the ordinary refluxing method they obtained 2.90% of ash-free lignin/

lignin containing 4.53% of methoxyl and 2.70% nitrogen. On the basis of the comparable yields of ash-free lignin, they concluded that there is no difference between the two methods. Judged after correction for crude protein, however, the first method would appear less efficient than the second in reducing lignin yield, which is very unlikely. Judged on the basis of the absolute methoxyl content of the lignin fraction the first residue probably contains much less true lignin than the other residue, so that it is very probable that the first residue contains much more non-protein contamination than the second one.

It may be noted at this point that comparisons on the basis of methoxyl content, the most important characteristic of lignin, have been overlooked by several workers investigating the application of lignin determinations in nutritional and pasture studies. Manning and De Long (1941) comparing several methods, used the absolute methoxyl content of the lignin fraction as an index of the purity of the lignin, but did not appear to attach particular importance to it at first. Later Sowden and De Long (1949a,b) found that the conclusions regarding the purity of lignin fractions and the amounts of absolute lignin present, drawn from quantitative studies of the ultraviolet/

ultraviolet absorption spectra of the prepared lignins, agreed well with those drawn from their methoxyl contents. Forbes and Garrigus (1950b) realised that in digestibility trials comparison of the ash-free lignin of food and faeces was unsatisfactory and they started to investigate the quality of the lignin fraction by determining both nitrogen and methoxyl contents. This led them to have less confidence in the present methods of determining lignin and pointed to the need for extended research on the subject.

1.3.2.4 Interference due to Ash. In the majority of cases ash-free lignin is recorded without information as to ash content of the original crude lignin. In wood analysis some workers record the whole lignin fraction as its ash content is very low. When nitrogen and/or methoxyl are determined in lignin they are often recorded as percentages of the lignin residue (Manning and De Long 1941; McDougal and De Long 1942, 1948a, b,c), but workers who realise the high ash content of the lignin of young plants often record them as percentages of the ash-free lignin, (Phillips et al, 1939, Sowden and De Long, 1949a,b). Over 40% of ash in the lignin fraction of young plants has been recorded by the latter workers and Phillips and Goss (1936b) found great variability in the ash content of straw lignin (3.42 - 9.05%) so that it is not possible to make a constant correction for the ash in lignin. The reasons for these great variations in ash content are not clear.

Sowden and De Long (1949b) recorded figures obtained with 3 samples of forage and two methods of analysis. The percentage of ash in the lignin fractions varied from 17 to 33 and was higher with one method of analysis that with the other; there were also variations between the different samples. It is clear that the calculation of nitrogen or methoxyl/

methoxyl percentages in the whole lignin fraction rather than in the ash-free lignin , is of doubtful value.

In the case of alkali lignin such as that prepared from young forage plants by Bondi and Meyer (1948) the ash content is only about 1% or less. This may indicate that nearly all the ash in acid lignin is a contaminant, but this supposition needs further support.

13.2.5. Other Sources of Error (i) Filtration

Davis and Miller (1939) claim that after the final treatment with dilute acid filtration should be carried out within 30 minutes. If the solutions is allowed to cool, it is difficult to filter and wash and the results obtained ~~were~~ are too high and should be discarded. Armitage et al (1948), however, found that the error due to cooling was negligible, and McDougal and De Long (1942) claimed that allowing samples to stand for half to one hour after the final refluxing facilitates filtration. These different views are most probably due to the short contact time with 72% sulphuric acid used in the Davis and Miller procedure, re-precepitation of partially degraded cellulose in colloidal form possibly occurring.

McDougal and De Long (1942) used naphthalene as/

as a filter-aid in a sintered-glass crucible, as described by Mueller and Herrmann (1926). A filtering mat is formed by adding a solution of naphthalene in alcohol to water: after filtration, the naphthalene is sublimed with steam so that the precipitate is not contaminated. In 1948, Armitage et al called attention to serious errors resulting from the use of alundum crucibles in the final filtration, and recommended Gooch crucibles to reduce the variability of replicates. The Gooch crucible has its limitations however if the lignin fraction is to be further analysed. Wise (1947) recommended the use of two ashless filter papers of practically the same weight instead of alundum crucibles. After filtration through both papers, then washing and drying the precipitate is weighed on the upper filter paper, using the lower paper as a counterbalance on the other pan of the balance. Manning and De Long (1941) recommended the use of Whatman No. 50 filter paper. Filter aids, such as 'hyflo super-cell' have also been used (Ellis et al 1946) and may be useful when determining ash-free lignin. It is possible that the filter-aid may retain contaminants, particularly if these are colloidal. This is likely during the main treatment and subsequent operations in strong acid methods. Perhaps

Perhaps most of the filtration difficulties are due to the colloidal behaviour of lignin, which may still be a source of error.

(ii) Temperature of Drying samples. McDougal and De Long (1942) using the Manning and De Long (1941) procedure studied the effect on lignin yield and quality, of the temperature at which fresh succulent plants were dried. They compared (a) drying at room temperature (b) drying at 60°C in vacuo, (c) drying at 105°C. The yield of lignin was higher following the high temperature drying particularly at 105°C and the authors concluded that insoluble artefacts are formed at high temperature, possibly by condensation of lignin with proteins and carbohydrates. They did not think that soluble lignins in the fresh material were converted to insoluble ones at high temperatures, although they admitted that the factors appeared to be complex. When the fresh material was washed with water to remove soluble substances, the yield of lignin after drying at 105°C was very much less than without the initial washing. The air-drying of washed material gives a still lower yield than drying at 105°C after washing. They concluded that the interference was largely due to water-soluble constituents. With the lower temperatures of drying the nitrogen content of the/

the lignin fraction was much less, but the methoxyl percentage was usually greater. In all cases when higher temperatures were used, the absolute methoxyl in the lignin was higher, so the authors concluded that some carbohydrate containing methoxyl may have condensed with the lignin. This is however, unlikely in a lignin determination procedure which involves a lengthy pretreatment (3hours boiling with water + 3 hours boiling with 1% hydrochloric acid) which is adequate to remove 'labile' or ester methoxyl present in certain carbohydrates (Phillips and Goss,1935, Phillips et al, 1939). The presence of methylated carbohydrates (containing firmly bound methoxyl) in plants has not yet been demonstrated (Phillips, 1946 p.345).

Ellis et al (1946) confirmed the effect of initial drying temperature on the yield of ash-free lignin from young barley clippings, but in mature tissue, (hay) the difference was small and possibly insignificant. Thomas and Armstrong (1949) studied the effect of drying temperature on the faeces from different types of feeding, using the Norman-Jenkins procedure (corrected for crude protein) and drying at 105°C, 60°C and room temperature. They found little difference between drying at 60°C and at room temperature but drying at 105° produced a small increase/

increase, which was probably significant. They recommend that in digestibility studies faecal material should be dried below 60°C. The great difference between fresh foods and faeces in their reaction to drying temperature is regarded as being due to the removal of soluble materials during passage of the food through the digestive tract.

1.3.3. Reproducibility Although the procedure for determining lignin in wood gives reproducible results this is not the case with food-stuffs and young plants. Armitage et al (1948) noted ~~that~~ the unsatisfactory agreement between replicates in lignin determinations and compared clover residues obtained after their pretreatment with the corrected lignin obtained after further treatment with 72% H_2SO_4 etc. There was excellent replication in the pretreatment residues (0.220, 0.220, 0.225 and 0.230) but using alundum crucibles for the final filtration the ash-free lignin varied from 6.6% to 14.15% . When alundum crucibles were replaced by Gooch crucibles the agreement between triplicate determinations of ash-free lignin was satisfactory, the maximum deviation in five different materials being between 0.6 and 7.1% of the mean.

Ellis et al (1946) stated that using their procedure with samples ranging from 0.5 to 2.0g., the ash/

ash-free lignin values agree to within 2 mg of each other, so that in samples containing 5% ash-free lignin, the maximum deviation from the mean may be up to 8%. Mean values with mean deviations as high as 16% have, however, been recorded (McDougal and De Long, 1948a) so it appears that in individual determinations the maximum deviation may be far greater than 16%.

Ellis (1949) published a statistical reproducebility study for the Ellis et al method (1946) using Timothy hay, immature grass and sheep faeces, analysed by different laboratories. Six replicates were run for each sample in each laboratory, and the standard error of the mean calculated; highest and lowest values were also given. There was a greater difference between laboratories than within a single laboratory. Hay samples gave the most uniform results and only in one case was there a statistically significant difference (at the 1% level) between two laboratories. With immature grass four out of fifteen comparisons were significantly different. Ellis concluded that the lignin method is reasonably satisfactory as regards reproducibility but recommended further work partifularly with immature plants. Inspection of his data shows that the maximum deviation reached with immature grass was 16% of the mean./

mean.

Forbes and Garrigus (1950b) using the same procedure in large scale digestibility trials with grasses, observed that the ash-free lignin from duplicate samples may vary widely, but the absolute methoxyl content is close. As a result a high ash-free lignin is associated with a low methoxyl percentage. They also noticed that differences between duplicate determinations of ash-free lignin are usually great if the two analyses are not made at the same time, and they concluded that the method still fails to remove other organic matter from the lignin residue.

These findings show clearly the danger of relying on reproducibility data based only on determinations of ash-free lignin. The ash-free residue may be satisfactorily reproducible, so long as the arbitrary steps of the determination are carried out precisely, but this does not prove the accuracy of the method. Some lignin may have been removed and various impurities may remain. The lignin residue, like the crude fibre fraction, may be reproducible, although consisting of a mixture of components.

1.4.1 Digestibility of Lignin

Workers using different methods and animals have claimed that lignin is indigestible, (Dietrich and Konig (1871); Poloheimo, 1925; Rogozinski and Starzweska, 1926; Crampton and Maynard, 1938; Naumann, (1940), Ellis et al, 1946; Ferguson, 1942, 1948; Swift et al 1947; Forbes and Garrigus, 1948, 1949, 1950a) or of only very low digestibility, (Konig and Becker, 1918; Hale et al, 1940; Lou, 1941; Ferguson, 1942). Other workers have obtained variable results; Lancaster (1943) has recorded both high negative and high positive digestibilities, whilst others have reported only positive digestibility (Rubner, 1928b, Bondi and Meyer, 1943) or negative digestibility (Forbes and Garrigus, 1950b). The data of Druce and Willcox (1949) and Rutledge and Common (1948) suggest that lignin is practically indigestible, but other figures of Rutledge and Common (1947) obtained with the same technique (Norman-Jenkins corrected for crude protein) suggest slight digestion. McCall et al (1943,1944) found that lignin digestibility varies considerably. These variable results are due to the fact that the recovery of lignin in the faeces depends on (a) the method of analysis, (b) the method of expressing the lignin residue (ash-free or corrected) and (c) the type/

type of food, (more discrepancies are observed with immature plants.

All the methods which have been used are open to criticism in some respect but the limitations have not always been immediately apparent. Frequently a new method of determination has appeared promising in biological work with animals, but later, when applied on a wider scale, it has been found to be unsatisfactory and its use abandoned. Thus Crampton and Maynard's method (1938) was abandoned by Ellis, Matrone and Maynard (1946) as unreliable and a new method evolved by them was supported by the experimental results of Swift et al (1947), Forbes and co-workers (~~1946~~, 1947, 1948, 1950a) and Ellis (1949). In 1950, however, Forbes and Garrigus (1950b) showed that this new method is biologically unsatisfactory for the lignin ratio technique and the quality of the lignin residues obtained is unsatisfactory, differing significantly in food lignin and faeces lignin. All the methods which have been evolved in the last two decades, particularly for nutritional work, are based on the assumption that the procedure which produces the lowest yield of lignin residue is preferable and represents a step forward. This assumption is not totally valid, particularly if acid hydrolysis is employed in the pretreatment or alkaline enzymatic/

enzymatic reagents are used.

According to McAnally (1942) if the lignin product obtained has not the same composition as the lignin "in situ", it does at least represent the lignin quantitatively, and digestion of the lignin will result in diminution of the yield of the substances isolated by the strong acid. This view is unacceptable, as the product is unlikely to represent the whole lignin, and the composition of the product is not constant.

Louw (1941) using the same method as McAnally, for veld grasses, stated that the errors in the figures for the lignin in the feed and in the faeces would cancel each other out to a certain extent, making the digestibility figures still reliable. If this is so, the method will suffice to give a measure of digestibility, which is a quotient, but it will not provide a measure of 'digestible lignin' which necessitates a knowledge of the absolute lignin content. Moreoever, the error in the determination of lignin in foods is probably greater and more variable than in the determination in faeces. Bruce and Willcox (1949) comparing Crampton and Maynard's method with that of Norman and Jenkins, found that the differences between them were much less with faeces than with foods, and it has already been

pointed/

pointed out that drying temperature may affect food lignin to a greater extent than faecal lignin.

The biological treatment which a food normally receives during digestion is in fact a substitute for the pretreatment of the faecal material in the lignin determination procedure and as it is efficient in removing soluble materials which interfere with lignin during the strong acid contact it accounts very well for the findings of Bruce and Willcox and Thomas and Armstrong mentioned above. Bondi and Meyer (1943) used the procedure of Kalb (1932) which involves no pretreatment except ether extraction for oven-dried forage crops and recorded very high digestibilities for lignin. These results are no doubt incorrect owing to high figures for the ash-free lignin in the food.

Straw pulp and wood pulp are being increasingly used for the feeding of ruminants. Sodium hydroxide is mainly used for delignification but several other chemicals can be employed to increase the palatability and digestibility of lignified material (e.g. $Ca(OH)_2$, Na_2S, KOH, ClO_2). The wood pulps are essentially cellulose and have high feeding value but are less palatable than straw pulps. The usual practice in this country is to soak the straw in 10 times its weight of alkaline solution (1.5% NaOH) for 12 - 20 hours/

hours, and then washing thoroughly with water (Watson 1941, 1949 p.170, Ferguson, 1942, 1943). The product from wheat straw with 15.1% lignin and 54.21% crude cellulose (cellulose and cellulosan) contains about 13.6% lignin and the rest is mainly crude cellulose (63.87%) and other carbohydrates. The straw loses 20% of its dry weight by this treatment i.e. 4.2% lignin and 15.8% of other soluble materials. It appears that the increase in digestibility of the pulp cannot be attributed only to removal of part of the lignin since the percentage of lignin in the pulp is similar to that in the original straw and the pulp has lost 15.8% of other soluble materials, mostly carbohydrates of high digestibility. Perhaps the effect of the treatment on cellulose itself and the mechanical changes in the structure of the plant cells are more important (Watson, 1941); in this connection, Trautmann and Asher (1941) found that unless cellulose from onion skin is treated with acid or alkali, it will not show any sign of digestion in the large intestine of ruminants.

It has been recorded (Schneider, 1947 p.265) that straw boiled with water has a higher digestibility than the original straw and in this case the percentage of lignin in the product would increase rather than decrease. The effect is very likely physical/

physical rather than due to delignification. On the other hand Prjanischnikow and Tomme (1936) delignified rye straw with chlorine dioxide and found a marked increase in the digestion of the nutrients by rabbits. The digestibility of fibre increased from 15 to 79% and that of pentosans from 23 to 81%. As a result of the treatment the straw showed a marked fall in lignin content but little, if any, change in the fibre, pentosans or nitrogen-free extractives. The marked increase in digestibility was attributed to the removal of lignin but it may perhaps have resulted from a change in the nature of the 'holocellulose' in the pulp produced by the oxidising agent.

Kormsoikov (1940) reported that treatment of straw with 2.53 parts of a 1% solution of lime for 24 hours followed by leaving 10 days for self-neutralization, was a more practicable method for obtaining straw pulp, with reduced cost.

The relative values of 'crude fibre' and "lignin" as measures of digestibility are still uncertain. Although high negative correlations between crude fibre and the digestibility of dry matter or organic matter have been recorded by McKeekan (1943), Lancaster (1943) and Phillips and Loughlin (1949) only a low correlation (-0.46) which was insignificant was reported by Thomas and Ibbotson (1947). Thus

the/

the crude fibre content is not a reliable index of digestibility in all cases. The correlations recorded for lignin content and organic matter digestibility, viz. -0.978 (Lancaster 1943), -0.954, -0.959 (Phillips and Laughlin 1949) suggest that lignin may be a better index of digestibility than the crude fibre content. Lancaster (1943) examined the digestibility of 17 different foodstuffs by sheep and calculated the following regression of organic matter digestibility (Y) on lignin content of the dry matter (X):- Y = 94.04 - 2.95X. Forbes and Garrigus (1950a) obtained the equations: Y = 100 - 4.71X for steers and Y = 100 - 5.24X for sheep grazing different forage crops in a total of 70 digestibility trials. As data for lignin are still limited the prediction of organic matter digestibility from the lignin content is not yet practicable on a large scale. Moreover the two regression equations for sheep given above show great differences, perhaps due to the method of determination of lignin as well as to the types of food examined.

1.4.2 Lignin and the Ratio Technique for Indirect determination of digestibility.

The ratio technique for indirect determination of digestibility was introduced by Bergeim (1926). If an indigestible substance is well mixed with the food and fully recovered in the faeces, it is possible to compute apparent digestibilities from a knowledge of the percentages of this substance in food and faeces and the percentages of the various nutrients, without quantitative measures of food and faeces. A simple mathematical equation is used:

(1) Apparent digestibility of nutrient n = 100 - 100 × $\frac{\text{L in food} \times \text{n in faeces}}{\text{L in faeces} \times \text{n in food}}$

where L is the percentage of the reference substance and n is the percentage of the particular nutrient. Heller (1928) and Rathnow (1938) used ferric oxide as reference substance but Knott et al (1936) and Hale et al (1940) found it unreliable for ruminants and Bruce and Willcox (1949) found it unsuitable with rabbits. Errors are almost certain to arise from irregular distribution of the iron oxide throughout the food during its passage through the digestive tract.

A silica ratio was proposed by Gallup and Kuhlmann (1931) but later (1936) after a critical study/

study, they stated that naturally occuring silica may be appreciably metabolized and eliminated through other channels. Bruce and Willcox (1949) however, obtained a satisfactory recovery with two rabbits (97.1 and 104.5%).

As lignin appeared to be practically indigestible, it was suggested as a natural marker, and Hale et al (1940) used a lignin ratio to asses the rate of passage of nutrients from the rumen as well as for determinations of digestibility with cows. They found that the lignin ratio technique was superior to the iron ratio, and recommended its use for studying changes in the rumen. In ordinary digestion trials, however, the recovery of lignin was between 76.3% and 105.1% so introducing an appreciable error into the calculated digestibilities. When feeding hay at a low level (10 lb) they found close agreement between digestibilities determined by the lignin-ratio method and by the conventional method, but at a high level of feeding (20lb and 30 lb) digestibilities determined by the lignin-ratio were much lower.

Ellis et al (1946) recommended their modified method for use in the lignin-ratio technique and supported it by data from digestibility trials with cows, sheep and rabbits. Comparing the digestibilities obtained/

obtained by lignin-ratio and conventional methods in only one out of 30 cases was there a difference in nutrient digestibility significant at the 1% level and in only 5 out of the 30 were differences significant at the 5% level. The daily variations in the lignin percentage of sheep faeces was shown to be remarkably small, so these authors recommended three or four days collection of faeces as being quite adequate. Their method of analysis was modified so as to give a minimum lignin residue and for use in the lignin ratio technique this is likely to be an advantage provided the food lignin fraction is completely recovered in the faeces. They realised that their method might not determine the whole of the lignin present.

The results of Ellis et al (1946) were confirmed for sheep by Swift et al (1947) using mixed feeds and by Forbes et al (1946) with clover-timothy hay, and for steers and sheep by Forbes and Garrigus (1947, 1948) using grazed pasture. The latter workers use the method as a means of determining the food intake of the grazing animal. They found the average recovery of the lignin consumed by steers in seven trials to be 102 ± 7%. Although this recovery is variable they considered the lignin ratio technique to be more accurate than any other method/

methods available to determine the dry matter intake of grazing animals. (e.g. 'dry matter ratio' and 'simultaneous dry matter ratio' methods) To obtain the dry matter intake by the lignin ratio techniques the faeces are collected quantitatively to determine the total lignin content. From a knowledge of the percentage of lignin in the grass, the amount of grass consumed is calculated. This method can give accurate results provided the percentage of lignin in the fraction of the grass which is eaten does not differ from that in the uneaten fraction. In the case of sheep which leave the more lignified stemmy parts of the plant grazed, the differences may be great and even if the recovery of the lignin is 100%, this selection would lead to an underestimate of the dry matter consumed.

In 1950, Forbes and Garrigus (1950a) extended the application of the lignin ratio method to enable the determination of both the digestibility of the pasture and the dry matter intake of the grazing animals. The same authors (1950b) using steers and sheep, obtained lignin recoveries of 106 ± 5.8% and 111 ± 8.7% respectively, indicating a significant negative digestibility of lignin. They found that correcting the lignin for crude protein improves the results, but slight negative digestibilities still persist/

persist. In the sheep experiments some wood shavings from the bedding were eaten and this would account for the additional recovery of lignin in the faeces. As this did not occur with the steers, it is more likely that the lignin fraction from the faeces was more contaminated than that from the food. In fact in their experiments the faeces lignin had a higher nitrogen content than the food lignin, and it is also possible that the faecal lignin contained more non-nitrogenous contaminants.

Hale et al (1947) attempted a mathematical procedure to correct for discrepancies arising in lignin-ratio digestibilities when the lignin is appreciably digested. For calculating the digestibilities by lignin ratio, they use a formula similar to that mentioned above, assuming the lignin to be indigestible. Then applying these calculated digestibilities they calculate the pounds of digestible crude fibre, digestible nitrogen-free extract, digestible cellulose and digestible 'other carbohydrates' in 100 lb food, using the conventional system of partitioning the crude carbohydrate fraction and Crampton and Maynard's system (lignin, cellulose and other carbohydrates by difference).

The digestibility of lignin itself was then calculated by the following formula

(2)/

(2) % Digestibility of lignin =

$$100 \times \frac{(\text{lb crude fibre digested} + \text{lb N.F.E. digested}) - (\text{lb of cellulose digested} + \text{lb of other carbohydrate digested})}{\text{pounds of Lignin in hay}}$$

Since the sums of the crude fibre + N.F.E. is equal to the sums of the cellulose + Lignin + other carbohydrates any differences in the 2 fractions represented in the numerator of formula (2) would be due to digestibility of the lignin.

They pointed out that in formula (2), the error in calculating the digestibilities of the nutrients involved would be of the same magnitude, so that the difference between the 2 fractions in the numerator of formula (2) would remain essentially unaltered.

After calculating the digestibility of lignin in this manner, they calculated the indigestible lignin in 100 parts of the food and then used this instead of the original lignin content of the food obtained by analysis, to recalculate the digestibility of each nutrient by formula (1) given previously, (page 95).

An example was given comparing digestibilities obtained by formula (1) before correcting for digestible lignin/

lignin, with those obtained after such correction and recalculation, and with those obtained by the conventional method. Apparently the example supports the validity of their equations and assumptions, and appears to justify the double fractionation of the 'crude carbohydrate' to gain the advantage of accuracy in the lignin ratio technique.

Unfortunately, examination shows that their assumptions are incorrect (see Chapter IX) so it is concluded that there must be errors in their data or calculations. If lignin is assumed to be indigestible and the digestible nutrients are obtained by formula (1) the difference between the 2 fractions in the numerator of formula (2) must be zero, whatever the actual digestibility of the lignin as determined by the conventional method. Formula (2) should always give a value of zero for lignin digestibility as this has been assumed.

1.4.3 Lignin Breakdown in the Animal Body

Little work has been done on this subject. Phillips et al (1929) and Csonka et al (1929) found that 'alkali lignin' from corn cobs fed to cows or dogs caused an increase in the benzoic acid excreted as hippuric acid in the urine. Some of the results are given in tables (2) and (3) below.

Table (2)+ Lignin Feeding Experiments with Dog, Showing Effect on Hippuric Acid (Benzoic acid) Elimination.

Meat Fed Daily (grams)	Lignin added to Meat Diet (grams)	Total Benzoic Acid Eliminated Daily (grams)
150	None	0.357
150	25	0.648
150	25	0.879
150	25	0.676
150	25	0.795
150	25	0.810
150	None	0.359
150	None	0.339
None	None	0.207
None	25	0.705

In two feeding experiments with cows, the average daily output of benzoic acid increased from 32.34g to 45.08g. and from 62.12g. to 64.85g. Only 63.3% of the methoxyl content of the alkali lignin added to the food was recovered in the faeces (table 3). In the case of dogs the amount of benzoic acid was almost doubled (from about 0.35g to about 0.70g.) and 80 to 86.3% of the methoxyl content of the/

Table (3)+ Methoxyl Group Balance in Metabolism Experiments

Experimental Animal	Fore Period Daily Methoxyl			Experimental Period (5 days)				
	Intake in food (g)	Output in faeces (g.)	% Loss	Methoxyl in Lignin added (g)	Total Methoxyl output in faeces (g.)	Increase over Normal output due to lignin (g)	Loss of Methoxyl in lignin added	
							grams	%
Dog	None	None	-	13.00	10.40	10.40	2.6	20.0
Dog	None	None	-	13.48	11.67	11.67	1.81	13.3
Cow	249.5	77.8	68.8	210.80	211.00	133.20	77.60	36.7

+ Data after Phillips et al (1929), Csonka et al (1929)

the alkali lignin in the food was recovered in the faeces. With cows, the difference in the benzoic acid excretion was possibly not significant for one experiment and the methoxyl estimation in faeces was by difference, as the food of the cow normally contains methoxyl groups. The results with dogs appear to be more conclusive, as the meat diet was found to contain no methoxyl. These workers also conducted an 'in vitro' experiment incubating alkali lignin with fresh material from a cow's stomach and they observed a loss of methoxyl.

Brigl and Pfahler (1929) found that addition of alkali lignin to the basic diet of sheep and rabbits increased benzoic acid output in the urine. The increase was equal to 8.0% of the weight of lignin added in the case of sheep and 5.2% in the case of the rabbit. Willstatter lignins prepared from straw, when fed to a rabbit led to an excretion of benzoic acid which was only 1% of the lignin fed, and alcohol lignin from hay caused the greatest percentage of benzoic acid increase. This indicates that of lignin isolated by different methods may behave differently and results obtained with isolated lignin may not necessarily apply to lignin 'in situ'.

Pazur and De Long (1948) studying the effect of the lignin content of dry clover at various stages of/

of maturity on the urinary excretion of aromatic acids by sheep, concluded that with young clover the excretion of hippuric and benzoic acids is three times as much as with the most mature clover. Their results were based on lignin determined by difference (Crampton and Whiting, 1943) and they assumed that lignin is only excreted in the urine as benzoic and hippuric acids and that no other plant constituent can give rise to these products. There is no guarantee that these assumptions are correct and the indirect method used to determine lignin is less satisfactory than direct determination. Also there were no control animals. The results are based on the amounts of aromatic acid excreted per 100 grams of lignin fed, but if calculated as the absolute amounts excreted from the foods at different stages of growth the differences are not great and possibly not significant. Excretion of aromatic acids in the urine may be fairly constant whatever the stage of growth of the clover.

1.4.4 Pasture studies The general conclusion from studies of pasture at different stages of growth is that the lignin percentage increases with age - Norman and Richardson (1937) and Patton and Gieseker (1942) found a gradual increase and similar results are recorded for barley and oats by Phillips and Goss/

Goss (1935) Phillips et al (1939), Drapala et al (1947) and for oats by McDougal and De Long (1948c). The nitrogen content both in the original plants and in the lignin decreases with age. Apparently there is a negative correlation between lignin and nitrogen. Louw (1941) studying changes of lignin and other constituents in veld grasses in S. Africa and their digestibility at four stages of growth (monthly intervals) found that lignin contents do not rise considerably (9.9 - 11.4%). Lignin in the crude fibre of plants and faeces averaged 3.5% and 10% respectively, the remainder being natural cellulose. On the other hand, Armstrong et al (1950) noticed differences between legumes (lucerne and trefoil) and grasses (perennial ryegrass, cocksfoot and tall fescue). In the former the rise in lignin percentage was gradual but in the latter, the rise was gradual only up to the flowering stage after which there was a more marked increase. The herbs (Burnet and Yarrow) were similar to the grasses. Their results confirm that the composition of crude fibre is variable even within a single species (Norman 1935, Louw 1941, Ferguson 1943). Between the middle of April and early July these plants species showed no considerable differences in lignin percentage at corresponding stages of growth; the authors expected that/

that mature lucerne or inferior grasses might have a higher lignin content. The lignin content of the crude fibre of legumes was three times as much as that of the grasses; the recovery of lignin in the crude fibre of legumes (relative to the percentage in the original dry matter) was higher than in the crude fibre of grasses. It seems that the nature of the lignin differs in different species, particularly the solubility in extractant. Lignin is more easily extracted by dilute alkali from grasses than from wood. The high lignin content in the crude fibre of legumes may explain the observed lower digestibility of the crude fibre as compared with that of grasses.

Phillips et al (1959) found that after pretreatment, the recovery of absolute methoxyl in acid lignin was relatively low with young plants but increased with more mature plants. They believed the unrecovered methoxyl to belong to carbohydrates containing firmly-bound methoxyl. These methylated carbohydrates were suggested to be the source of lignin formation. Phillips (1946 p.345) however indicated that the existence of such methylated carbohydrates had never been demonstrated.

The observed methoxyl percentage in lignin increases with the age of the plant from about 2 to 17% in graminese. The figures for mature graminese approach/

approach that of acid lignin from soft woods. Although Bondi and Meyer (1948) indicated that legume lignins contain about 5% methoxyl, higher figures (about 10%) have been recorded by the same workers (1943) for the ash-free lignin obtained from the crude fibre of forage legumes (10.2% in Lathyrus and 7.56% in Vicia). They found that the corresponding figures for the lignin of the whole food are much lower (Bondi and Meyer 1943). Forbes and Garrigus (1950b) recorded 9.77% methoxyl in the ash-free lignin from forage alfalfa, using a method of determination involving efficient pretreatment. This shows how the method of determination may seriously affect the lignin quality, and illustrates that the methoxyl content of plant lignins requires much further study.

Sowden and De Long (1949a,b) using two different procedures (Manning-De Long and Crampton-Maynard) compared the qualities of the lignin obtained and found great differences between the two methods: the forage lignins had low methoxyl and nigh nitrogen contents and were only of 50% purity, relative to wood lignin, as measured by absorption spectra, ~~against wood lignin.~~ They concluded that the current methods for determining lignin in young plants are so inaccurate that conclusions regarding the digestibility of lignin, based on the use of such analytical procedures, are of questionable validity.

C H A P T E R II

General Analytical Methods

2.1. Nitrogen Determination

The Kjeldahl micro-and semi-micro procedures recommended by Ogg et al (1948) were used as these are suitable for aromatic substances. Details are given in the Appendix (page 1). Bruel et al (1947) showed that N/100 NaOH may change in strength by interaction with the narrow orifice of the micro-burette, or merely on ageing, so the use of boric acid solution and mixed indicator was preferred.

Boiling tubes (ca.50ml) were first used for digestion but the recovery of tryptophane nitrogen was only about 80%. This was not unexpected since when heating by sand bath or direct micro-flame, the fuming and boiling of the acid was irregular, and the clearing time variable; in some cases the volume of the acid diminished appreciably, upsetting the proportion between the sulphuric acid and selenium in the mixture. To overcome this difficulty, a suitable air condenser for the top of the boiling tube was made from a tapering wide glass tube with a narrow tube outlet at the upper end, somewhat similar to an inverted thistle funnel (see Fig.1 Appendix p.32). The taper permits the use of the condenser in boiling tubes of varying diameter. Using this condenser and/

and heating with a direct flame, complete recovery of tryptophane nitrogen was obtained. In later analyses 30 ml micro-kjeldahl flasks heated by micro burners were employed. In some cases micro-digestion in 300 ml Kjeldahl flasks was employed in order to reduce sampling errors, suitable fractions of the digests being taken for microdistillation.

The distillation apparatus used was that described by Pregl (1945, p.80) and the time of distillation was determined by the volume of distillate.

The mixed indicator recommended by Ogg et al (1948) (methylene-blue + methyl red) ~~1945~~ was compared with that of Ma and Zuazaga (1942) (Bromocresol Green + methyl red) and it was found that with the former thechanges in colour at the end point are more distinct and more sensitive to the dilute acid, giving better reporducibility and more accurate results. Blank determinations in which the digestion flasks loosely stoppered gave results (ca 0.09ml N/100HCl) comparable with others in which the flasks were left open, indicating that contamination from the atmosphere was of no significance.

The blank varied from day to day but it was not always necessary to titrate it with N/100 HCl as its colour (after distillation) could be used to indicate the end points of other determinations.

Micro/

Macro-determination of nitrogen (see appendix, page 5) was carried out using the same reagents as in the micro and semimicro determinations; 20 ml conc. H_2SO_4 were used for digestion, 20 ml boric acid for absorption of the ammonia, and 0.05N HCl for titration. In the macro-distillations a glass tube (approx 15 cm × 1 cm) partly immersed in the boiling solution, ensure regular boiling.

When the lignin fraction was filtered through asbestos or glass wool in a Gooch crucible, the whole residue, together with the filtering medium, was taken and it was necessary to use macrodigestion (with 10 or 20 ml H_2SO_4) to ensure regular boiling and clearing. It was also found more accurate and convenient in these cases to use macrodistillation and micro-titration rather than microdistillation of a fraction of the digest.

When the digest contained asbestos or glass wool (which would render boiling irregular) it was transferred to the distilling flask through a funnel plugged with glass wool.

2.2. Methoxyl determination Both semi-micro and macro determinations by the Zeisel method were made, details of the procedures being given in the Appendix (page 5). In this method the alkoxy group is split off as the alkyl iodide by boiling the sample with hydriodic acid; the volatile alkyl iodide is carried over by a stream of carbon dioxide, absorbed in a receiver and determined gravimetrically or volumetrically. In the volumetric procedure employed, the iodide is collected in a solution of bromine, sodium (or potassium) acetate, and acetic acid and after adding formic acid to remove excess bromine the iodate produced is determined iodometrically titrating with thiosulphate solution and using starch indicator. For multiple determinations this method is quicker than the gravimetric method employing absorption by an alcoholic solution of silver nitrate to form AgI, $AgNO_3$, a double salt decomposed by dilute nitric acid with the production of AgI. The gravimetric method fails with substances containing sulphur, and besides methoxyl and ethoxyl groups, other lower alkyl groups bound to oxygen also yield AgI (Pregl, 1945, p.152). In foods and lignin residues such interfering substances are likely to be present.

2.1/

2.2.1 Reactions

(a) Release of alkyl iodide

(1) $HI + R_1.O.R \rightarrow R_1OH + R_2I$ (R = alkyl group.)

(2) $HI + R_1.O.CH_3 \rightarrow R_1OH + CH_3I$

(b) Absorption of alkyl iodide

(3) $CH_3I + Br_2 \rightarrow CH_3Br + IBr$

(4) $IBr + 3H_2O + 2Br_2 \rightarrow HIO_3 + 5HBr$

The acids produced (equation 4) are neutralized by the strong sodium acetate.

(c) Removal of Excess Br_2

The reaction appears to be:

(5) $Br_2 + HCOOH \rightarrow 2HBr + CO_2$

(After addition of HCOOH to bromine water, CO_2 was detected coming over, and Br^- was detected in the solution by $AgNO_3$ reaction, and bromoform production). Neutralisation of the excess of formic acid by NaOAC was found to be necessary.

(d) Determination of Iodate

This is by the iodometric method, titrating with thiosulphate after adding KI and H_2SO_4.

(6) $KIO_3 + 5\ KI + 3H_2SO_4 \rightarrow 3K_2SO_4 + 3I_2 + 3H_2O$

$6Na_2S_2O_3 + 3I_2 \rightarrow 3Na_2S_4O_6 + 6NaI$

2.2.2 Semi-Micro determination: The procedure, reagents and apparatus described in the A.O.A.C. Methods of/

of Analysis (1945 p.762) were used, with the following modifications:-

(i) A solution of 5% thiosulphate was used in the trap instead of water (recommended by Pregl (1945 p.152).

(ii) 0.05N or 0.025N $Na_2S_2O_3$ was used.

(iii) The apparatus was modified to facilitate manipulation and permit the introduction of sodium thiosulphate solution into the trap without contaminating the tube (C) leading to the receiver (see Fig. 2 Appendix page 33). The joint just above the trap also facilitated washing and drying of the apparatus.

It was found unnecessary to use two receivers as in the A.O.A.C. apparatus; provided no solution was carried over from the first receiver to the second, during the course of the distillation, the titration value for the solution in the second receiver was exactly equal to a reagent blank. Owing to the narrow dimensions of the first receiver, however, a surge of CO_2 such as sometimes occurred at the commencement of distillation, was liable to lead to loss by splashing. The two receivers of the A.O.A.C. apparatus were therefore replaced by a single one (D) of more suitable dimensions made from a test tube widened in the middle but having a narrow lower part.

This/

This design increased the capacity of the receiver and permitted the use of 10 to 25 ml solution without splashing, so that the apparatus could be used for a wider range of determinations (from micro to sub-macro scale).

(iv) The micro-burner used to heat the hydriodic acid digestion mixture was provided with a wire gauze; a direct flame was found to render the trap hot allowing some phenol to be carried over to the receiver. When this happened it was found to interfere, reducing the apparent alkoxyl content.

(v) The digestion time was extended to 1½ hours.

In some lignin fractions containing little methoxyl, 100 or 150 mg were needed for analysis and the volume of hydriodic acid was increased to 7½ or 15 ml. In other cases when the amount of material permitted, 50 mg were taken for analysis (instead of 20mg) in order to increase the accuracy.

2.2.2.1. Testing the procedure: For this purpose Vanillin dried over sulphuric acid under vacuum was used; this substance was preferred as it is one of the degradation products of lignin containing a guaiacyl radicle.

(i) Hydriodic acid and thiosulphate trap. A.R. hydriodic acid (S.G. 1.7) was used and 35 - 60mg vanillin, with a digestion time of 1½ hrs. When the/

the hydriodic acid was not redistilled and thiosulphate was not used in the trap, duplicate analyses gave values of 20.6 and 20.7% methoxyl (theoretical value, 20.4), indicating a recovery of 101.2% ±0.3. Although this recovery was satisfactory, the blank titration was high (0.70ml 0.05N $Na_2S_2O_3$). The hydriodic acid was then redistilled from a 500ml Claisen flask fitted with thermometer and water condenser, and the first and last distillates not boiling at 127°C were discarded. Using the redistilled hydriodic acid and a 5% solution of thiosulphate in the trap the recovery of methoxyl from vanillin was 100.1% and the blank titration was only 0.1 ml and remained constant from day to day. This procedure was therefore adopted and both the stock hydriodic acid and the freshly distilled portion were stored in the dark in an atmosphere of CO_2 to minimise oxidation and separation of iodine.

(ii) Digestion time - Using the same procedure but only digesting for 1 hour 10 minutes the recovery of methoxyl was low (96.6%). This was confirmed in macro-determinations using an apparatus similar to that described by Doree (1947 p.597); only 73.4% recovery was obtained after 1 hr. digestion, but after 1½ hrs. digestion the recovery was 98.1% ±0.01. Prolonging the digestion beyond 1½ hrs. gave no

further/

further significant increase; a lignin fraction from seeds hay showed a methoxyl content of 14.05% ±0.05 after 1½ hrs. digestion and after digestion for a further ½ hr. the amount of thiosulphate required for titration (0.18ml) was no more than that required for a reagent blank.

It was concluded that in both macro- and semi-micro determinations, it is desirable to digest for 1½ hrs.

(iii) Formic acid reaction - Since formic acid can act as a reducing agent it appeared that the excess present after the removal of bromine might reduce some of the standard thiosulphate used in titration. A blank reagent was therefore prepared from bromine and potassium acetate in glacial acetic acid solution, and to this was added formic acid and a strong solution of NaOAc to drive off the bromine. Then 20 ml 0.05N KIO_3 were added, and the mixture titrated with 0.05N thiosulphate. The titration (18.6ml) was practically identical with that required (18.7ml) for the direct titration of 20ml 0.05N KIO_3. When the addition of NaOAC solution was omitted, however only 4 ml of thiosulphate were required. It was concluded that excess of formic acid does not interfere in the usual procedure, and that the iodate formed from the alkyl iodide is quantitatively determined.

2.2.3 Macro-determination The same reagents and procedure as in the semi-micro determination were used, employing 20 ml hydriodic acid, and 20 ml receiving solution. A macro-apparatus similar to that described by Dorée (1947p.397) was satisfactory but the manipulation was found to be somewhat time consuming. Modifications similar to those in the semi-micro apparatus were therefore introduced (see Appendix p.34). Digestion was carried out in an ordinary distilling flask (50ml) the side arm of which was bent upward to allow condensed vapours to run back into the flask; the air condenser and trap were similar in shape and size to those in the semi-micro apparatus and were connected by rubber stopper to the flask and by a short length of rubber tubing to the delivery tube above. An ordinary boiling tube of about 50 ml capacity was used as receiver. This apparatus had the advantage of only two joints to be made air tight and the long neck of the distilling flask gave an increased condensing capacity which was an advantage with the larger volume of boiling mixture.

2.3. Acid Lignin Determination

Modifications of the methods of Ellis et al (1946) and Armitage et al (1948) were employed and are described in the Appendix (p 12 and 15). A new method was also developed and is described in Chapter VII (page 373). In this chapter only general analytical methods are dealt with and the various filtrations techniques which were tried in lignin separations are therefore described.

A sintered glass filter stick as described by Ellis et al (1946) was found to choke after a period of use and the filter-aid hyflo-supercel did not appreciably speed the filtration. A more practicable filter stick was made from a perforated porcelain disc fitted into the wide mouth of a thistle funnel, covered with a disc of Whatman No.50 filter paper and held firmly in position by covering with a large piece of parachute cloth tied to the sides of the funnel by nylon thread. A reversal of the air flow quickly restored the speed of filtration with this stick and facilitated complete washing.

Filtration through Whatman No.50 paper was found very satisfactory as this paper would resist the suction of a water-pump and its smooth surface allowed the residue to be easily washed out.

It was found that suction could be applied to this/

this filter paper when folded in an ordinary glass funnel provided the upper edge of the filter paper was kept in position (e.g. by inverting a smaller funnel onto the inner surface of the filter paper). This procedure was quicker and more satisfactory than a filter stick, or even a Buchner funnel with the same paper (Fig.4). The pretreated plant material could be dried with a filter paper and then removed practically quantitatively, particularly when large samples (5 - 10g.) of plant material were used. When small samples (1g) were pretreated, they were usually transferred for digestion with 72% H_2SO_4, and even scratching the surface of filter paper to ensure quantitative removal did not interfere with the results, as any cellulose removed was readily hydrolysed. The wet residue could also be quantitatively removed from the filter; this was usually done after pepsin digestion in the method of Ellis et al, after 5% HCl hydrolysis in the method of Armitage et al, and after the first filtration in the final treatment of the former procedure. Using this method of filtering it was possible to prepare lignin from single samples as large as 10g. so facilitating qualitative examination of the lignin residues.

For filtering large pretreated samples a Buchner funnel fitted with a muslin disc below a Whatman No.50 paper/

paper was found to be very suitable.

Sintered glass or Gooch crucibles were used to filter the final lignin residues. When a final hydrolysis was used, filtration through both types of crucible was easy. If the final hydrolysis was omitted, however, filtration through sintered glass crucibles was slow, particularly with large samples so a primary filtration was made through Whatman No.50 paper as described above, the wet residue being transferred to the crucible for secondary filtration and washing.

When the 72% H_2SO_4 or fuming HCl used in the main treatment was filtered without dilution the Gooch crucible was the only means of quantitative filtration. An asbestos mat was satisfactory in some cases, but a glass wool mat (prepared from glass wool cut into 3 - 4 mm lengths) was found to be the most satisfactory and rapid filtering medium.

A Gooch crucible fitted with a disc of Whatman No.50 paper was suitable for filtering small amounts of prepared lignins (alkali or ester lignin); sintered silica crucibles, also served the same purpose, but were found to be slow in filtering final lignin residues.

C H A P T E R III

Preliminary Examination of Lignin Analysis Techniques

As indicated in the introduction, nearly every stage in lignin determination procedures has been criticised, and as no detailed reproducibility study has been reported preliminary work was devoted to an examination of the sources of error involved and the qualities of the residues obtained at each stage, were noted, as well as the effect on the final lignin residue.

3.1. Reproducibility and sources of error. The procedure employed in this study was that of Ellis et al (1946) which combines enzymatic and chemical methods of pretreatment, and has been fairly widely used in nutritional work. The reproducibility study was not intended to assess the value of this particular method but rather to indicate the sources of error likely to be encountered in the isolation of acid lignin.

3.1.1 Experimental A sample of seeds hay dried at 100°C was examined by the method of Ellis et al (1946) with only slight modifications: for extraction ordinary Soxhlet thimbles were used, for filtration, Whatman No.50 filter paper and laboratory-made filter sticks were employed and the final lignin/

lignin residues were filtered through ~~in~~ Gooch or sintered glass crucibles. In a few cases hyflo-supercel filter-aid was used in separating the lignin residue.

The rate of syphoning of the ethanol-benzene solution in the Soxhlet apparatus was usually about twice per hour.

After pepsin digestion the plant material was collected on Whatman No.50 filter paper, washed free from HCl then washed with alcohol and ether and finally dried. In order to study the reproducibility in any single stage of the procedure, a large homogeneous sample was carried through the preceding stages and then subdivided into small samples to be used as replicates.

The experimental scheme is illustrated in Table 4, below and the results given in Tables A1 to A5 are summarised in Table A6, (See Appendix P 47).

3.1.2 Results and discussion

Experiment 1 Ethanol-Benzene Extraction The first samples were extracted with the Soxhlet apparatus set as for ether extraction and the syphoning rate with ethanol benzene was very slow (Table A1). With later samples ~~(Table A 5 b)~~ higher syphoning rates were obtained.

a. Residue From the data in Table A1 the deviation between/

Table 4. Experimental Scheme of Reproducibility Study

Expt.	Starting Material	Treatment	Determinations	Appropriate table containing results.
1	Original oven dried material	Ethanol-Benzene extraction. (a) low syphoning rate (b) high syphoning rate	(a) Residue and Nitrogen (b) Residue and ash	A 1
2	Residue after Ethanol-Benzene extraction	(a) Pepsin digestion only (b) Pepsin digestion + 5% H_2SO_4 hydrolysis	(a) Residue and Nitrogen (b) " " "	A 2
3	Original oven dried sample	(a) Ethanol-Benzene extn. only (b) " " + pepsin digestn. + 5% H_2SO_4 hydrolysis	(a) Residue (b) Residue and Ash	A 3
4	Residue after Ethanol-Benzene extraction and pretreatment	72% H_2SO_4 contact + 3% H_2SO_4 hydrolysis	Lignin residue, ash-free lignin, nitrogen and ash	A 4
5	Original sample	The whole procedure (a) with small samples (b) with large samples	(a) ash-free lignin ± ~~total~~ lignin residue (b) Lignin residue, ash-free lignin, nitrogen and ash	A 5a A 5b

between replicates as judged by the standard error of the mean, maximum deviation and coefficient of variation, is likely to be more than the error in sampling.

Close observation of the Soxhlet apparatus during operation revealed slight differences in the mode and rateof syphoning owing to differences in the height of the syphon and to interference from air bubbles in the syphon. These appear to be the principal causes of deviation. Extraction curves obtained in later experiments revealed that the time and rate of syphoning affect reproducibility; the longer the time of extraction and the higher the rate of syphoning the closer are the replicates, due no doubt to the nearer approach to complete extraction.

(b) Nitrogen content. A sample of the combined residues from experiment 1a contained 83.1% of the original nitrogen, indicating a removal of about 1/6 of the total N during ethanol-benzene extraction.

(c) Ash content. The residues from samples 9, 10, 11 and 12 (Table A1) were very similar in quantity the difference between duplicates being less than 1.5%. The difference between corresponding ashes however were greater, being 6.8% for samples 9 and 10, and 4.6% for samples 11 and 12. This was probably due to slight differences in the nature of the/

the residues. It may be noted that the samples yielding the greater residues gave the higher ash percentages so that the percentages of ash-free residues will be in closer agreement than the crude residues.

The observed differences in ash percentage (4.6% and 6.8%) are not likely to be due to experimental error which in weighing 50 mg ash, should not exceed 0.8% (the sensitivity of the balance used was 0.4mg per scale division).

On the basis of the average figures for samples 9, 10, 11,12, the residue represents 90.5% of the ash in the original sample, so that about one tenth of the ash is removed in the ethanol-benzene extraction.

Experiment 2a Pepsin-HCl digestion In this experiment a bulked sample of ethanol-benzene extracted residue was used after thorough mixing. The residue after pepsin digestion was filtered, washed with alcohol and ether, dried and weighed.

(a) Residue The deviation between replicates (Table A2) is much less than in the residues from ethanol-benzene extraction, the maximum deviation being 2.08% and the coefficient of variation 1.5% whilst the corresponding figures for ethanol-benzene residues were 5.3% and 2.8%. In this stage the agreement is satisfactory and the error negligible. To avoid errors due to particles floating or sticking

to/

to the sides of the vessel during incubation, wide mouthed, glass stoppered bottles were used and almost completely filled with the reaction mixture.

(b) Nitrogen content The wide variation in nitrogen percentage and absolute nitrogen content shows a decided difference in the nature of the residues, and illustrates the fact that enzymatic treatments are not so easily standardised as purely chemical treatments.

There is an average loss of 23.11% of the dry weight during this treatment, and a loss of 2.36% (6.25 × 0.376*) crude protein so that 20.75% of non-protein material is removed, no doubt by the effect of the warm acid solution. This fraction is easily soluble carbonydrate and does not contain true cellulose.

* 0.376 is the difference between absolute nitrogen contents in ethanol-benzene residues and pepsin HCl residue.

Experiment 2b. Pepsin - HCl digestion + 5% H_2SO_4 hydrolysis

(a) Residue When the final residue is expressed as a percentage of the residue after pepsin-HCl treatment only, the error is less than when expressed as a percentage of the starting material (Table A2). Despite this accumulation of error the deviations arising in these two stages are still less than the deviations/

deviations in the ethanol-benzene extraction process. In the 5% H_2SO_4 hydrolysis there was a loss of 40% of the dry weight of the pepsin residue corresponding to 28.6% of the original dry matter. Allowing for small amounts of nitrogenous material and ash, (0.6% and 1.9% of the D.M. respectively), about 1/4 of the organic matter is removed in this stage. This fraction would be largely carbohydrate, but may contain some lignin as claimed by some workers.

When the 5% H_2SO_4 filtrate was allowed to stand for a day a very slight brownish humin residue settled down and became more noticeable on prolonged standing. This has been observed by other workers and there is some doubt regarding the nature of the sediment. Some workers (Harris and Mitchell, 1939, Cohen and Harris 1957) believe it to be lignin.

(b) Nitrogen content The variation in the nitrogen content of the final residue is less than in that of the residue after enzymatic treatment only, probably because the H_2SO_4 hydrolysis has an effect complementary to the pepsin digestion. The variation is small because the amount of nitrogen is small and judged by nitrogen content the residues are practically identical in nature.

(c) In the ethanol-benzene plus pepsin-HCl treatment 70% of the nitrogen of the original dry matter is removed/

removed, and the whole pretreatment (including 5% acid hydrolysis) removed 87% . Armitage et al (1948) obtained up to 95.3% extraction of the nitrogen of clover (21.1% crude protein) using 2½ hrs. ethanol-benzene extraction and trypsin digestion for 18 hours followed by 5% HCl hydrolysis for 1 hr. This may suggest that the Armitage et al procedure is more efficient but the great difference in nitrogen content between the seeds hay (0.71%) used here and the clover (3.38%) used by Armitage must be considered. Although a high percentage of the nitrogen content of clover is removed by the procedure of Armitage et al, yet the absolute nitrogen content left in the pretreated residue (expressed as a percentage of the original sample) is over 10 times as great as that left in the seeds hay pretreated by the method of Ellis et al (1% and 0.09% respectively). The percentage extraction of nitrogen is probably dependent not only on the method but also on the nitrogen content of the plant material itself and perhaps indirectly on the lignin content since a nitrogen fraction may be associated with the lignin in the pretreated residue.

Experiment 3 Ethanol-benzene extraction alone and followed by pepsin digestion and H_2SO_4 hydrolysis

This experiment was mainly to determine the reproducibility in the whole pretreatment but reproducibility of/

of the ethanol-benzene extraction was also determined to see how far the deviations in the ethanol-benzene residue would affect the final residue. The ash content was used to indicate the homogeneity of the residues.

(a) Residue The agreement between replicates (Table A3) is only slightly less than in Experiment 2.

The average residues obtained in Expts 2 and 3 (41.39 ± 0.473 (4) and 40.76 ± 0.505 (4)), are not significantly different (t = 0.92 and P = 0.4).

It is concluded that starting with a homogeneous sample of ethanol-benzene residue (Expt.2) does not diminish the variation in the final pretreated residue. In other words the high variations found in the ethanol-benzene residues are not maintained in the final residues. This is perhaps because some material which escapes extraction by ethanol-benzene is extracted in the last two steps of the pretreatment. (This possibility was later substantiated by evidence.)

(b) Ash-content Deviations between the ash contents of four samples were negligible (for about 20 mg ash) and it seems that the nature of the residues is not different. On an average 62.9% of the ash in the original dry matter is removed in the whole pretreatment.

Combining the results of Expts 2 and 3, the nature/

nature of the pretreated residue in replicate experiments is practically identical and errors are likely due to mechanical losses occurring during the operations, and to any errors in sampling the starting material.

Experiment 4 Reproducibility of the Main Treatment and 3% Acid hydrolysis A large sample was extracted with ethanol-benzene and treated with pepsin and 5% H_2SO_4. After digestion with 72% H_2SO_4 and dilution 7.5 times the first filtrate produced a brownish humin sediment on standing, but the final filtrate did not. The nature of the residue was investigated and is discussed in a later chapter.

(a) Lignin Residue The variation in the lignin residue is (Table A4) higher than the permissible experimental error, but the ash-free lignin shows satisfactory agreement. The ash content of the crude lignin is very variable but the organic fraction is much more constant. It appears that the higher the lignin content the higher is the percentage in the lignin and although the crude lignin residues differ between replicates, yet the organic matter may be of the same nature. The close agreement between replicate values for ash-free lignin indicates that the main treatment with 72% H_2SO_4 and the final hydrolysis with 3% H_2SO_4 are not responsible for deviations in the results and the large deviations recorded/

recorded by some previous workers (McDougal and De Long 1948c, Ellis et al, 1946, Armitage et al 1948) may be due to cumulative errors, or filtration errors, as indicated by Armitage et al (1948).

(b) Ash content This is high and very variable and appears to be correlated positively with crude lignin content. The recovery in the crude lignin of the ash of the starting material exceeds 100% in one case and is between 64 and 92% in 3 cases. It is possible that H_2SO_4 is partly retained by the lignin residue, removal of some of the ash occurring concurrently and the ash retained may not be of constant composition; a chemically retained or insoluble fraction may be constant but a labile fraction may also be physically retained. Washing the final residue may affect the amount of physically retained ash and may account for ash variations.

(c) Nitrogen content The absolute nitrogen content is less variable than the nitrogen percentage, as the latter is affected by the variable lignin residue. Differences in the absolute nitrogen content may be within the limits of experimental error. The relatively constant nitrogen content of the ash-free lignin suggests a uniform composition.

The average recovery of nitrogen in the lignin residue is 90.1% of the nitrogen in the starting material/

material (pretreated). The retained nitrogen is probably of a different nature from that removed. Phillips (1939) found that straw lignin contained 56.5% of the nitrogen of the pretreated residue and concluded that the nitrogen retained may not be protein in nature. The higher recovery from seeds hay may be due to the pretreatment being more efficient in removing the less resistant nitrogen.

The absolute amount of nitrogen retained in the lignin in this experiment is 14.5% of that in the original dry matter. As the lignin fraction may be practically indigestible the nitrogen in it will have no nutritive value so the amount present is of considerable importance especially in the case of mature grasses and hays where the lignin content is high and the nitrogen content low.

The nitrogen content of the ash-free lignin obtained here is within the limits recorded by Bondi and Mayer (1948) for "alkali lignin".

Experiment 5. Reproducibility of the whole procedure.

(a) Small samples (Expt. 5a) Both the crude lignin and ash free lignin residues (Table A5a) are more variable than in Expt. 4, but the mean lignin residue (average of 3 samples) is not significantly different from that in Expt.4 or in Expt. 5b (large sample).

The/

The ash-free lignin of two samples using filter aid is slightly higher than the average of the other three (without filter aid) and although the difference is not significant the use of filter aid possibly prevents small mechanical losses.

The magnitude of the deviations is within the limits recorded by other workers but nevertheless seems high when judged on the basis of the deviations in the pretreated wt residue (Expts. 2b and 3b) and the deviations following 72% acid contact and prolonged hydrolysis (Expt. 4). Though Armitage et al (1948) considered 7% deviation to be permissible in such a lengthy procedure, there is reason to believe that this error is more than can be accounted for by normal experimental or mechanical losses. It appears that some unknown factors may be involved.

(b) Large sample (Expt. 5b) In order to reduce the error from losses and to determine with greater accuracy the ash and nitrogen contents larger samples were employed.

The agreement between duplicate values for lignin residue, ash free lignin and corrected lignin (Table A5b) is satisfactory and there is no great deviation in ash and nitrogen contents, although the latter is distinctly higher than the previous experiment (5a)

The/

The percentage of ash-free lignin is lower than in Expts 4 and 5a but the differences are not significant. It is noted that when the pretreated residue was dehydrated with alcohol prior to strong acid contact, the alcohol filtrate was brownish and turbid and on heating precipitated humin-like substances, some of which solidified and blackened; the black residue was insoluble in 72% H_2SO_4. This was not observed with the small samples possibly because the alcohol percolated through them quickly and the relatively small amounts of material removed was not so obvious. It is possible that the alcohol washing removes some interfering substances or material insoluble in 72% H_2SO_4 and so diminishes the yield of lignin or increases its variability.

In the case of the large samples, efficient ethanol-benzene extraction was employed (syphoning in Soxhlet every 3 minutes, instead of every 30 minutes as in the extraction of the small samples) and this may also have the effect of lowering lignin yield.

The error possibly introduced in the alcohol washing, may account for some variations in the yield of pretreated residue, but this is probably of small magnitude as the weight of pretreated residue is relatively large. As the weight of lignin is much smaller the substances removed by alcohol washing may have/

have a correspondingly greater effect on the variability of the lignin residue.

3.1.3 General conclusions

(1) The residues from ethanol-benzene extraction may show appreciable variation but this does not persist after subsequent operations.

(2) After organic solvent extraction and pretreatment agreement between replicates is satisfactory, although the nature of the residues may vary slightly. The reproducibility of the residues after 72% H_2SO_4 treatment and final hydrolysis is satisfactory when starting from a homogeneous pretreated sample, but when combined with organic solvent extraction and pretreatment great variation in the residues results. This seems to be because of an error introduced between the pretreatment and subsequent operations, and may be related to the "soluble substances" removed by alcohol washing after the 5% H_2SO_4 hydrolysis.

(3) Results expressed in terms of the whole lignin residue appear to be the least reliable because of the high and variable content of ash. Figures for ash-free lignin are more reliable but may differ from those expressed as corrected lignin; indicating that the error due to nitrogenous material in the lignin fraction is not proportional to the nitrogen content/

content.

(4) The observed humin precipitate in the 5% H_2SO_4 hydrolysates, the alcohol-soluble residue after this treatment, and the humin precipitate in the first filtrate after diluting the 72% H_2SO_4 (before hydrolysis) substantiate the conclusion that there is an analytical error in the lignin determination procedure, and show the necessity for studying the nature of these residues, and their relation to the dilute acid hydrolysis, 72% H_2SO_4 treatment, and final hydrolysis.

3.2. The Reliability of the Principles of Lignin Determination

3.2.1 Contact with 72% H_2SO_4 at low temperature. By using a freezing mixture of 2 parts ice + 1 part conc. H_2SO_4 + 1 part conc. HNO_3 (Perry 1941 p.26) temperatures down to -14°C were obtained. Plant material was placed in a small wide mouth reagent bottle along with the previously cooled acid and the temperature inside the bottle was maintained between -10 and -12°C by changing the freezing mixture when necessary. A mechanical stirrer was employed.

Experiment (i) 0.35 g. of straw pretreated by the method of Ellis et al (1946) were treated with 20 ml 72% H_2SO_4 at -10° to -12°C for 6 hours and portions were withdrawn at intervals and filtered through glass wool in tubes immersed in the freezing mixture.

After 2 hours the solid particles still retained their original shape and the solution was viscous. Portions of the filtrate were examined as follows:-

1) Dilution with 7 volumes of water gave a flocculent white precipitate which floated on the surface and gave a positive reaction in the I_2-H_2SO_4 test for cellulose.

2) Warming to 20°C gave no precipitate.

3) A portion was left 1 hour at 20°C, then one half was diluted with 6 volumes of water and the other half/

half with 38 volumes of water (to produce 3% w/wH_2SO_4).

In each case a small amount of brownish precipitate settled down; it gave a negative reaction in the cellulose test.

4) A portion was left 1 hour at 0°C, then diluted as before (3).

In each case a flocculent white precipitate was obtained which gave a positive reaction in the cellulose test. No brownish precipitate was observed. After 6 hours reaction the mixture was still viscous, and solid particles of plant residue still persisted.

This experiment was repeated and a residue of undissolved plant material was again obtained after 6 hours reaction, contrary to the claims of Hilpert and others. (Hilpert and Littmann, 1934,35, Hilpert and Peres, 1935). The failure of the filtrate to produce a precipitate on warming to 20°C was also contrary to the findings of Hilpert.

A flocculent precipitate was reported by Hilpert and his co workers to be produced on diluting the cooled mixture, but here it was also observed after holding the cold filtrate for 1 hour at 0°C. Hilpert indicated that the precipitate was carbohydrate, representing "either an intermediate product or a component particularly stable to acid" and Hilpert and/

and Littman (1935) gave it the formula $2C_6H_{10}O_5.H_2O$. The precipitate obtained in Experiment 1 was clearly cellulose ($C_6H_{10}O_5$) or cellulose dextrine which were 'dispersed', the hydrolysis being impaired at low temperature so that reprecipitation was possible (D.P.* over 30 anhydro sugar units, Crampton, 1948). The cellulose 'dispersed' by 2 hours contact below -10°C was no longer reprecipitable after one hour at 20°. The brownish precipitate is not of cellulose origin, and as it was not observed before raising the temperature, it was probably produced by the interaction of the strong acid and some soluble material during the period at 20°C. The formation of this brown residue was not noticeable at low temperature.

From the results of this experiment and the criticism of many other chemists, (Norman 1937, P152 McAnally 1942), it appears that Wilpert's claim regarding the complete dissolution of straw in cold 72% H_2SO_4 is not justified. The brown precipitate obtained by raising the temperature and diluting shows that the strong acid may interact with some soluble material to produce a soluble compound not of cellulose origin which precipitates on diluting.

* Degree of polymerization.

Experiment ii

Further tests were made at 0°C or in a refrigerator to study the conditions under which the brown humin residue is found. The results (Table 5) show that 2 hours contact at 0°C does not produce any humin residue, but 24 hours contact or retaining the filtrate at 0 - 5°C for 24 hours leads to the formation of a slight amount of brown precipitate.

The formation of the residue after 2 hours contact at 20°C, is probably the effect of the high temperature since at low temperatures, the reaction was slow and practically undetectable at 0°C, (2 hrs. contact).

Even after 24 hours contact with 72% H_2SO_4 at 20°C (or room temperature) however, no reprecipitation occurs until the acid is diluted so that it is not necessary to lower the contact temperature so long as the filtration of the mixture is carried out without dilution.

The strong acid may also disperse some associates without chemical change, these being precipitated by dilution, but filtration without dilution will eliminate all substances reprecipitated by dilution, and it appeared possible by this means to avoid a serious error in the accepted lignin determination procedure.

Table 5 Contact of pretreated straw with 72% H_2SO_4 at low temperature.
Expt. 11 (Section 2.1.)

No. of Treatment.	Treatment of Acid-plant mixture	Observation
1	6 hours at below -10° then 24 hrs in refrigerator	Diln. of filtrate produced white flocculent ppte. tinged with brown giving +ve cellulose test.
2	2 hours in refrigerator	Diln. produced only white flocculent ppte. which gave +ve cellulose reaction. When the filtrate was refrigerated (Ca 4°C) for 24 hours, diln. produced a white flocculent ppte. tinged with brown; most of the white ppte. remained suspended, the brown humin ppte. settling down. Cellulose test +ve.
3	24 hours in refrigerator	Diln. of filtrate gave same result as in No.2 treatment when the filtrate was refrigerated for 24 hours.

3.2.2 The effect of strong acid contact with some nitrogen compounds and carbohydrates.

Experiments were conducted to see the extent of the interference of N compounds and carbohydrates by the formation of "insoluble" condensation products. The interference of these substances may be quite different in the presence of plant material or isolated lignin as they may combine with lignin or be retained by it. This aspect is examined in Chapter V. With sugars, particularly fructose, Hilpert and Littman (1934) claimed the formation of "sugar lignins" under the conditions of lignin determination but in fact they used higher temperatures and longer contact times than are usually used in lignin determination. Other workers found that under the conditions of lignin determination, the formation of insoluble humin-like substances is negligible (See Chapter I Section 3) P61).

Experiment (i) Between 0.1 and 0.2g. of agar, pectin, gelatine, starch, dextrin and gum arabic were treated separately with about 12 ml 72% H_2SO_4 in closed tubes at room temperature (15-20°C). A mixture of these six substances was also treated in the same way. Portions were removed at intervals, diluted to 3% acid concentration and boiled in a water bath.

In/

In all cases the material dissolved and with agar, pectin and the mixed materials the solution was brownish in colour; the others were clear.

Portions diluted after 2 hours, 24 hours, 2 days and 5 days when boiled gave no ppte. The mixtures before dilution appeared to be true solutions; only agar, pectin and the mixed material gave dark solutions; the gelatine solution remained transparent and colourless, whilst the starch, dextrin and gum arabic solutions were yellowish-brown.

After 5 days the solution of the mixed materials was diluted to 3% (about 4 cc diluted 38 times) and boiled 2 hrs. then filtered through a prepared Gooch crucible; no increase in the weight of the Gooch was obtained.

Experiment (ii) Pure cellulose (ashless filter paper) was treated in the same way (about 0.4g. was added to 20 ml 72% H_2SO_4). After 2 hrs. complete dispersion was not achieved as some cellulose particles were sticking to the sides of the container. Even so, diluting a portion of the viscous acid mixture did not produce any reprecipition. Boiling the diluted acid also gave no residue. Similar results were obtained after 24 hours contact.

Experiment (iii) Fructose was recorded by Hilpert and Littmann (1934) and by Phillips and Goss (1938)/

(1938) to produce some insoluble residue so its behaviour with 72% H_2SO_4 was tested.

A - About 0.5g. fructose was treated with 10 ml 72% H_2SO_4 at 20°C for 2 hrs. and then left 24 hours at room temperature (below 20°C).

After 2 hrs. 3ml diluted to 3% acid concentration and boiled 2 hrs. gave no ppte. even on standing overnight.

Another portion was diluted 3 - 4 times, and heated in a water bath, When a bulky ppte. formed a few minutes after boiling; the mixture darkened and had a "caramel" odour.

After filtering through a sintered silica crucible the ppte. was mostly soluble in 72% H_2SO_4 (although some particles remained undissolved) and when this acid solution was again diluted a bulky brownish humin precipitate was produced.

After 24 hrs. contact the original mixture had become dark coloured but did not contain any precipitate. Dilution to 3% concentration followed by boiling gave a slight brownish precipitate. Dilution 3 - 4 times gave the same result as after 2 hours contact.

B - A solution of 0.5g. fructose in 10 ml 72% H_2SO_4 was divided into portions which were diluted with 3, 5, 6 or 10 volumes of water and heated in a boiling water/

water bath, for 1½ hrs. Humin formation occurred in all portions, the bulk of the precipitate decreasing as the dilution increased; in the first dilution (using 3 volumes H_2O) the residue was bulky and darkening occurred, whilst in the last dilution (10 volumes water) the solution became yellowish in colour and the precipitate was negligible.

Experiment (iv) Glucose was treated in the same way as fructose in Expt. iiiB using dilution with 1, 2, 5, 10, 15 and 38 volumes of water followed by heating. A bulky precipitate formed in the first portion, a slight precipitate in the second and third and none in the last three dilutions. Glucose in 72% H_2SO_4 kept up to 24 hours at room temperature did not produce any precipitate either in the strong acid or after dilution with 38 volumes of water.

Experiment (v) Xylose in 72% H_2SO_4 for 3 hours and for 24 hours did not produce any precipitate.

When the acid xylose solution was diluted 5 times with water, and heated in a boiling water bath, a bulky precipitate was obtained, which gave a furfural reaction (with 12% HCl). This precipitate dissolved in alkali solution, and when passed through an alkali extraction prodedure (Appendix p. 8) produced little residue; although it/

it resembled 'alkali lignin' in colour, solubility in alkali and reprecipitation by acid, heating the dilute acid precipitate did not produce flakes typical of true 'alkali lignin'.

Discussion and Conclusions

These experiments indicate that in short-time contact with 72% H_2SO_4 which is widely used, agar, pectin, gelatine, starch, dextrin, gum arabic, cellulose, fructose, glucose and xylose do not form insoluble products either in the strong acid or after dilution of the acid ~~of~~ 38 times, even if this is followed by boiling. Even after prolonged contact (24 hrs. at 15 - 20°C) no insoluble products are not formed except in the case of fructose which produces a little residue. The formation of large amounts of insoluble "caramelization products" occurs when the 72% H_2SO_4 is only diluted to a low degree and then heated; this practice is not usually employed in any method of determination, and the high yield of humin products obtained by Hilpert and Littmann (1934) with sugars and 72% H_2SO_4 may have been determined by the extent to which the strong acid was diluted before boiling, rather than by the time and temperature of contact. These "caramilization products" exhibit humin-like properties, dissolve in alkali and reprecipitate on acidifying. They are different/

different from lignin, mostly redissolving in 72% H_2SO_4; if formed from pentose they can produce furfuraldehyde; the coagulation in dilute acids does not resemble that of alkali-lignin. The small fraction insoluble in 72% H_2SO_4 resembles acid lignin in colour and insolubility.

From section 2.1 above it seems that other substances from the plant can produce insoluble humin materials in the presence of 72% H_2SO_4 under lignin determination conditions and a study of the behaviour of the strong acid filtrate after contact with the plant seems to be more useful than testing pure substances.

(See over for Table 6)

3.2.3 The Reaction of strong acid with cellulose

Although different acid mixtures and concentrations have been proposed, no comparative study of their efficiencies has been made. As pretreated plant material consists mainly of cellulose and lignin, the acid treatment is intended principally to disperse and hydrolyse the former in order to separate it from the latter. Since different plant materials may differ in the proportion and nature of their "cellulosic material" for comparison of different acid reagents and procedures pure cellulose was employed.

Experimental procedure The reagents examined are listed in Table 6 below. Fuming HCl was prepared with the apparatus used in the A.O.A.C. method for determining lignin, a current of dry HCl being passed through conc. HCl at about -4°C for 1 hr. The final density was not less than 1.2 at 0°C.

The proposed mixture 1 and Schwalbe's Mixture (1925) were prepared by dropping the H_2SO_4 into the HCl solution cooled down to -12°C to prevent loss of HCl gas. Whatman No. 42 Ashless filter paper was used as the source of standard cellulose. Portions of the mixtures were examined at ½ - 1 hr. intervals, 1 - 2 ml being diluted 7 - 10 times, well shaken and left to settle.

Discussion and conclusions.

1./

Table 6 Dispersion and degradation of Pure Cellulose in contact with strong acids

No. of treatment.	Acid or acid mixture	Temp °C.	Stirring	Filter paper	Remarks
1	72% H_2SO_4 (37.5ml)	Room below 20	Mechanical shaker	0.9g. (3×4 mm pieces)	Reprecipitation occurred on dilution after 1½ hrs but not after 2 hrs. Complete dispersion in 4 hrs.
2	do. 20ml	20	Occasional stirring	0.3g. (1.5×2 mm.)	1. In 3½hrs almost dispersed 2. " 4hrs complete dispersion
3	do. 20ml	Room	Mechanical shaker	0.4g. (1.5×2½ mm.)	1. In 2½hrs almost complete dispn. and no reprecipitation on dil. 2. In 3 hrs complete dispn.
4	do. 20ml	Room	Mechanical shaker	0.4g. (0.5 × 1.3 mm pieces)	1. In 1½hrs incomplete dispn. 2. In 2 hrs complete dispn.
5	conc. HCl (100ml) (S.G. 1.18)	Room	Mechanical shaker	0.7g. (3×4mm pieces)	1. Disintegration of the filter paper occurred without dispersion.
6a	Fuming HCL 50ml. Reaction in tightly stoppered vessel	Ice cold then room temp.	Gas bubbling until dispn. complete	0.5g. (3×4mm)	1. After 2 hrs reprecipitation occurred on dilution. 2. After 24 hrs no reppte.

Table 6 (continued)

No. of treatment	Acid or acid mixture	Tempt. °C	Stirring	Filter paper	Remarks
6b	Cellulose freshly dispersed in fuming HCl left in loosely stoppered container	Ice cold, then Room temp. (below 20)	Glass bubling until dispn. complete	0.59 (3×4 m.m.	After 24 hrs. reprecipitation of cellulosic material occurred.
7	Fuming HCl (50ml) Reaction in tightly stoppered vessel	do.	do.	do.	1. Diminishing reppte. up to 4 hrs. no ppte. after 5 hours.
8	Kalb's mixture (20ml) 2ℓ. HCl (S.G. 1.18) + 272 ml H_2SO_4 (S.G. 1.84) at -12°C	Room	Mechanical shaker	0.4g (1.5×3½mm)	1. Complete dispersion in 1 hr. 2. A ppte. occurred on dilu. after 6⅔ hrs but not after 8 hours or 24 hrs.
9	Proposed mixture 1, (60ml) ¼ ℓ. H_2SO_4 + 1 ℓ. conc HCl.	Room 18°C	Mechanical stirrer	0.7g. (3×4mm)	1. Complete dispersion in slightly less than 1 hr. 2. After 2 nrs. dilution produced reprecipitation.
10	do	do	Mechanical shaker	do	do
11	Proposed mixture 1, (20ml)	Room	do	0.4g. 1.5 × 3½mm)	1. Complete dispn. in 1⅓ nrs. 2. Reprecipitation occurred on dilution after 3⅓ hrs but not after 4⅓ hrs or 8 hours.

Table 6 cont.

No. of treatment	Acid or acid mixture	Temp. °C	Stirring	Filter paper	Remarks
12	Schwalbe's mixture (1925)(37.5ml) (30ml 72% H_2SO_4 + 7.5ml 14% HCl	Room	Mechanical shaker	0.9g. (3×4mm)	1. Complete dispersion in 1½ hrs. 2. Reprecipitation occurred on dilution after digestion for periods up to 24 hrs.
13	72% H_2SO_4 20ml and bubbling with HCl gas.	Room	Gas bubbling	0.4g. (1.5 × 3.5mm)	1. Up to 1½hrs, dispersion incomplete.
14	72% H_2SO_4 20ml bubbled with HCl gas for ¾ hr. at 0°C	Room	Mechanical shaker	0.4g (1.5 × 3.5mm)	1. Almost complete dispersion in 2 hrs. 2. Diln. after 2 hrs. produced no precipitate even after settling overnight.
15	do	do	do	0.4g (1.5 × 2.5mm)	1. Complete dispn. occurred in 2½ hrs. 2. No precipitate was produced on dilution.
16	do	do	do	0.4g (0.5 × 1.5mm)	1. Complete dispn in 2 hrs. 2. No ppte. on diln.
17	Poppoff's (1958) 20ml $ZnCl_2$-HCl mixture (100ml 37% HCl+40g. $ZnCl_2$ + 5-10ml H_2O	Room	Occasional stirring	0.4g. (2×3mm)	The mixture behaved as conc. HCl treatment 5, disintegrating cellulose to fine particles.
18	Proposed mixture 2 (1 vol. conc. HCl + 1 vol. 72% H_2SO_4 w/w) 20ml.	Room	Gentle stirring	0.4g. (2×3mm)	Dispersed cellulose quicker than 72% H_2SO_4, but reprecipitation occurred even after 12 hours.

1. The time necessary for complete dispersion of cellulose is shortest with fuming HCl, and increases in the following order:- (1)Fuming HCl - (2) Kalb's mixture - (3) Proposed mixture 1 - (4) Schwalbes mixture (1925) - (5) 72% H_2SO_4 saturated with HCl gas $\frac{3}{4}$ hr. at 0°C) - (6) 72% H_2SO_4. Conc. HCl and the $ZnCl_2$ -HCl mixture were found to disintegrate ~~and~~ cellulose but failed to disperse it.

2. Degradation of cellulose to a stage where it is not reprecipitated on dilution occurs most quickly with 72% H_2SO_4 and progressively more slowly with the following:- (1) 72% H_2SO_4 (alone or treated with HCl gas) - (2) Fuming HCl for 20 mins. at 0°C to disperse followed by retention at room temperature - (3) Proposed mixture 1 - (4) Kalb's mixture - (5) Schwalbe's mixture.

3. Although 72% H_2SO_4 is the most efficient reagent for the degradation of cellulose, dispersion is slow and in treatment (4), 2 hrs. were only just sufficient for this purpose. There is some doubt therefore, whether 2 hours at 20°C or below will be adequate in all cases, particularly with plant material where the natural cellulose is more resistant than the prepared cellulose of filter paper. It is highly probable that with this short time of reaction some plant cellusic material will remain undispersed/

undispersed and contaminate the lignin fraction as deduced by Manning and De Long (1941) from their quantitative data.

A further difficulty arises due to particles of cellulose sticking to the sides of the vessel even though mechanical stirring or shaking is employed and particles also adhere to the stirrer and so become less easily attacked by the acid.

The viscous nature of the 72% H_2SO_4 - Cellulose mixture after 2 hrs makes it necessary to dilute before filtering although dilution may not be desirable. The sulphuric acid reagent has the advantage of being easily prepared and used and can be stored without change in concentration.

4. Fuming HCl requires contact for 5 hrs. at room temperature to avoid reprecipitation. Phillips and Goss (1936a) found that after 6 hours contact at 8 - 10°C the lignin yield was too high, no doubt due to reprecipitation of cellulose. Although in the A.O.A.C. procedure contact is for 24 hrs. at 8 - 10°C, it seems possible to shorten the time using a well stoppered container at room temperature. Phillips and Goss found that 24 hrs. contact at 8 - 10°C and at 18°C showed no material difference in lignin yield and quality.

Fuming HCl, Kalb's and Schwalbe's mixtures and the/

the proposed mixture 1 all give in ½ - 1 hr. non-viscous solutions which can be filtered without dilution although evolution of HCl may occur during filtration, upsetting the equilibrium and possibly causing reprecipitation of cellulose.

4. Kalb's mixture and the proposed mixture 1 are easier than fuming HCl to prepare and store and could well replace fuming HCl. The proposed mixture 1 produces quicker hydrolysis than Kalb's mixture.

5. Saturating cold 72% H_2SO_4 with HCl gas gives no advantage.

6. A mechanical shaker (end over end) is preferable to occassional hand stirring, and to mechanical stirring in the case of HCl mixtures.

7. The structure of the cellulose considerably affects the dispersion time (cf. treatments 3 and 4) especially in the case of 72% H_2SO_4. The rate of hydrolysis by H_2SO_4 at 20°C or room temperature is greater than the rate of dispersion so that it is possible to have fragments of undispersed cellulose existing in a solution which gives no precipitate on dilution. It is therefore necessary to ensure that the final lignin residue obtained by the current method gives no cellulose fraction.

8. Treatment No.6b shows that any leakage in the case of fuming HCl (and by analogy H_2SO_4-HCl mixtures) would/

would lead to incomplete hydrolysis of cellulose and contamination of the lignin residue. To avoid errors from this source the application of a cellulose test before dilution is also necessary.

9. The failure of Poppoff's mixture even to disperse cellulose is perhaps due to a technical error in preparing the mixture; cooling it in a freezing mixture, or saturating it with HCl gas in the cold, may be necessary.

Table 7 Composition of Various Acid Reagents
(assuming no loss of HCl gas during preparation)

Mixture	H_2SO_4%	HCl%	H_2O%
1. Fuming HCl	0	41-43	59-57
2. Kalb's mixture	16.4	29.8	53.8
3. Proposed mixture 1	27.2	22.9	49.9
4. " " 2	37.2	15.0	47.8
5. Schwalbe's (1925) mixture	63.40	1.96	34.6
6. 72% H_2SO_4	72	0	28

10. The rate of dispersion of cellulose by H_2SO_4-HCl mixtures increases as the proportion of HCl increases and decreases as the proportion of H_2SO_4 increases. (The composition of these acid mixtures is given in Table 7). Neither of these acids alone can effect dispersion except at high concentration, but in mixtures where the individual concentrations are relatively low, each acid seems to catalyse the action/

action of the other. The fuming HCl on the addition of H_2SO_4 may have a major influence on the efficiency of the mixture. Secure stoppering of the fuming H_2SO_4-HCl reaction mixtures so as to lead to the development of pressure is essential.

11. With acid reagents liable to change in concentration, a blank test with standard cellulose is preferable to density measurement as the latter is rarely of adquate sensitivity. For example the evolution of all the HCl from Schwalbe's mixture may produce hardly any detectable change in density.

3.2.4 The application of a cellulose test in the reaction of materials with 72% H_2SO_4.

The degradation products of cellulose or cellulose dextrin give a bluish colour with I_2-H_2SO_4 and a violet or blue colour with I_2-$ZnCl_2$ reagent. (Norman 1937 p34, Hawk et al 1949 p.80)

Tests showed that starch and dextrin react with iodine but dilution with water does not remove the colour. In the case of cellulose, however, a dispersing and degrading agent is necessary before a reaction is obtained and the colour developed with soluble cellulosic material disappears on dilution.

(i) Tests with pure cellulose.

A mixture of 72% H_2SO_4 and filter paper was tested at intervals with both reagents. After 2 hrs. the/

the I-$ZnCl_2$ reaction was negative, but the I_2-H_2SO_4 reaction did not become negative until after 4 hours digestion.

As the I_2-H_2SO_4 reagent appeared to be more sensitive it was preferred for general use. It was also found that the 72% H_2SO_4 hydrolysate gave a positive reaction to this test although reprecipitation of degraded cellulose did not occur on dilution.

When using fuming HCl in a closed container a negative reaction was obtained after 6 hours and dilution did not produce any reprecipitation. It appears therefore that with both fuming HCl and 72% H_2SO_4 a negative reation with I_2-H_2SO_4 is a reliable indication that no reprecipitation of degraded cellulose will occur on dilution.

(ii) Tests with plant material.

A sample of pretreated straw (as in Section 2.1) was digested with 72% H_2SO_4 for 2 hrs. at 20°C with occasional stirring. A slight violet colour was obtained with I_2-H_2SO_4 indicating the presence of some cellulosic material. After hydrolysis of the residue with 3% H_2SO_4, however the reaction was negative indicating that the final hydrolysis in the method of Ellis et al produces further degradation of cellulose, and hydrolysis is necessary.

In a further test the mixture after 2 hrs. digestion with/

with H_2SO_4 at room temperature was filtered through glass wool but some of the plant material remained sticking to the sides of the original vessel.

(a) The plant material filtered off gave a negative reaction in the cellulose test but the material sticking to the side of the container gave a decidedly positive cellulose reaction. This material was redigested with fresh 72% H_2SO_4 for ½hr. and then nearly all the particles gave a negative reaction. After a further digestion for ½ hr. no particle was found giving a positive reaction.

The filtered digest was held at room temperature and examined at intervals and it was only after 4 hrs that a negative reaction was obtained, although diluting and boiling the digest immediately after the 2 hrs. contact produced a negative reaction.

It appears necessary in the reaction with 72% H_2SO_4 to redigest for a further one hr any particles adhering to the sides of the container. Increasing the contact time to 5 hrs. will not achieve the same result as it is necessary to suspend the particles in the acid, and this can only be done satisfactorily with fresh, non-viscous acid. With particles of plant material the resistance to digestion may be even greater than with pure cellulose, as insoluble encrusting lignin material also is present.

(iii)/

(iii) Tests with lignin.

In some cases the test was clear, but in others it was indefinite owing to the dark colour of the lignin itself. It is more practicable to test for cellulose in the acid-plant mixture, than in the prepared lignin as in the event of a positive reaction the time of digestion may be increased or fresh 72% H_2SO_4 may be used and the determination satisfactorily completed. The practice of digesting with 72% H_2SO_4 until a negative reaction was obtained with I_2-H_2SO_4, was used by Konig and Rump (1914).

3.2.5 A Study of Filtrates from the Reaction of Plant Material with Strong Acid.

In these experiments pretreated material corresponding to approx. 1g. of the original plant material was treated with 20 ml 72% H_2SO_4 or 50 ml fuming HCl. Portions of the acid-plant mixture were filtered and examined at intervals. The acid filtrate was examined without dilution inorder to study the properties of associates or contaminants. Filtering difficulties were encountered. Centrifuging proved unsatisfactory and although glass wool was useful at first, some of the glass particles passed into the filtrate and better results were obtained with sintered silica crucibles containing a layer of hyflo-supercel, or Gooch crucibles, the latter being/

being preferred. Sintered glass crucibles were unsatisfactory and with sintered silica crucibles the filtration of viscous H_2SO_4 was often very slow.

Experiments:

(i) A mixture of pretreated straw and 72% H_2SO_4 was left 2 hrs. at room temperature then centrifuged and the supernatant liquid syphoned off. On dilution 5 to 10 times a decided brownish precipitate was produced. The experiment was repeated with periods of contact with the acid varying from 2 hrs 20 mins. to 6 hrs. 50 mins. and in all cases the brown humin precipitate was obtained. The persistence of this precipitate suggested that it was not cellulosic in nature.

(ii) An H_2SO_4 filtrate diluted to 3% concentration was boiled for 2 hrs. under a reflux condenser. The precipitate resisted this treatment and became more granulated, leaving a very clear supernatant solution. It was filtered through Whatman No. 50 paper and examined as follows:
1. a portion was added to conc. HCl at 0°C and bubbled with HCl gas when it mostly dissolved; on diluting with water the brown humin precipitate again appeared.
2. a portion was dried in a steam oven, and became brownish-black in appearance and failed to dissolve in 72% H_2SO_4, colouring the acid only slightly yellow.

Acid/

Acid lignin prepared by the method of Ellis et al behaves in a similar manner.

5. a portion was air-dried on a filter paper and the paper and precipitate added to 72% H_2SO_4 when almost complete solution occurred.

4. The humin precipitate gave a negative reaction to the cellulose test.

It is concluded that the precipitate is not of cellulosic nature, but since it is precipitated when the strong acid is diluted, and resists boiling it will appear in the lignin residue although originally soluble in 72% H_2SO_4.

(iii) Portions of the original mixture of plant material and 72% H_2SO_4 were filtered through glass wool at intervals, and the filtrates were also examined at several different times as shown in Table 8. Dilution was 7.5 times as ~~that~~ in the 1st dilution used in the procedure of Ellis et al after 72% H_2SO_4 contact; in all cases the humin precipitate was formed.

Table 8. Times of digestion and dilution in Expt. iii

Filtrate No.	Time of contact with 72% H_2SO_4	Times of retention of filtrate before dilution. (hrs.)
1	3	0, 1.5, 3.5, 7, 21.
2	4.5	0.2
3	5.5	0, 1, 4.5
4	6.5	0, 3.5
5	10	0
6	24	0

(a)/

(a) The precipitate obtained from filtrate No.1 diluted immediately did not completely dissolve in fuming HCl, some turbidity persisting in the mixture; on diluting again the humin precipitate reappeared. Some of the fuming HCl mixture was left undiluted for 24 hours and a slight humin precipitate settled out. Although fuming HCl appears to produce some dispersion of the humin precipitate, 72% H_2SO_4 is probably more effective in dissolving it.

(b) Some of filtrate No.1 was kept undiluted at room temperature. After 24 hours the mixture became more brown in colour but appeared clear whilst after 48 hours the colour had further darkened, and distinct dispersed particles were observed. Dilution produced a humin precipitate.

Since a precipitate occurred in all the dilutions indicated in Table 8, the presence of a soluble substance in the strong acid which is precipitated by dilution is obviously not affected by the time of contact. The formation of a precipitate in the undiluted filtrate, however, requires a long period of time, little appearing in 24 hrs.

The precipitate is not of cellulosic nature, and solubility tests show that it does not resemble lignin.

As long contact (24 hrs.) with the strong acid did/

did not produce insoluble"reversion products", the source of error is associated only with the dilution operation.

As previous workers (Norman and Jenkins,1934a,b, Phillips and Goss,1938, Hilpert and Littmann,1934 and others)have studied the resultant of two operations viz. acid contact plus dilution and final hydrolysis the actual reason for the production of insoluble contaminants was not detected.

(iv) 1.05g. pretreated straw were treated with 40ml 72% H_2SO_4 at 20°C for varying periods of time and dilutions from 0.1 to 38 times were made as indicated in Table 9.

Table 9 Times of digestion and dilution in Expt iv

Filtrate No.	Time of contact with 72% H_2SO_4 (hrs)	Times of retention of filtrates before dilution(hrs)	Vols of water added
1	2	0, 2, 4	7.5 and 38
2	4	0, 2	7.5
3	6	0	(2, 3, 4, 5, (6, 7, 7.5, 8 (10
4	3.5	0	7.5
5	24	0	(0.1, 0.2, 0.3, (0.4, 0.5, 0.6, (0.8, and 7.5

In all cases a humin precipitate appeared but with dilution to 1.1 and 1.2 volumes the precipitate was largely dispersed and the "supernatant" solution turbid. It is clearly not possible to avoid precipitation/

precipitation by only a low degree of dilution. The production of the precipitate by very small proportions of water suggests that it may be the density rather than the concentration of the acid which is important.

Some of filtrate No.1 was diluted 38 times (giving 3% w/w H_2SO_4) and refluxed for 2 hours but the residue persisted after boiling, as before.

Some of filtrate No.4 was diluted 7.5 times and filtered through Whatman No.50 paper, then washed extensively to remove traces of H_2SO_4, before air-drying. The residue did not completely redissolve in 72% H_2SO_4.

It appears that drying produces some physical or chemical change in the particles reducing the solubility or dispersibility.

(v) The effect of relatively short periods of contact was examined with 0.6g. pretreated straw and 20 ml 72% H_2SO_4 at 20°C the treatment being as shown in Table 10. The negative test for cellulose given by the precipitate from filtrate No.1 indicates that even after only 1½ hrs. reaction no reprecipitation of dispersed and degraded cellulosic material occurred. Some of the precipitate was filtered through hyflo-supercel in a sintered silica crucible, treated with 72% H_2SO_4 and the filtrate collected. At first it appeared brownish but soon came clear, and after dilution and prolonged settling a slight brownish precipitate reappeared. Some of the original precipitate/

precipitate in the filter remained undissolved even after 2 hours acid contact.

Filtrate No.2 treated in the same way as No.1 gave the same results.

(vi) In this experiment still shorter periods of contact were employed. High dilutions (38 times)

Table 10 Times of digestion and treatment in Expt.v

Filtrate No.	Time of contact with 72% H_2SO_4 (Hrs)	Vols. of water added	Treatment of precipitate and observations
1	1.5	38	Brown humin ppte. gave negative cellulose reaction.
2	2	7.5	(a) Brown humin ppte. gave negative cellulose reaction
		38	(b) Refluxed 2 hrs. giving a turbidity soln. producing a humin ppte. on standing.
3	3	38	Brown humin ppte. produced.

gave turbid solutions which settled only slowly unless refluxed (Table 11). Refluxing coagulated the precipitate and gave a clear supernatant liquid. It was thought that perhaps the precipitate formed directly on diluting to a high degree might be in a more or less colloidal form passing through the filtering medium and this was tested with Whatman No.50 filter paper.

The results indicate that the precipitate after 45 minutes digestion is mainly of cellulose origin, "Amyloid"*

* the flocculent reprecipitated cellulosic material

Table 11 Times of digestion and treatments in Expt vi

Filtrate No.	Time of contact with 72% H_2SO_4 (hrs)	Vols of water added	Treatment and observation
1	45	7.5	A very bulky flocculent white ppte. tinged with brown was obtained and gave a positive cellulose reaction.
2	120	7.5	a) Diln produced a distinctly brown humin, ppte. of only about one sixth of the volume of that from filtrate No.1 It gave a negative cellulose test reaction.
		38	b) Refluxing 2 hrs gave a brownish ppte
3	180	38	This was filtered through Whatman No.50 paper immediately after diln. and the filtrate remained clear even after ½ hrs refluxing and then allowed to stand. Brownish marks were evident on the filter paper.

"Amyloid", mixed with the brown ppte. which alone is obtained after longer contact times. Contamination from reprecipitated cellulosic material does not occur after 2 hours contact with 72% H_2SO_4 although in Davis and Miller's procedure (1939) where the contact time was 45 - 60 minutes, reprecipitation of "amyloid" was thus encountered, Davis stated that delay in the final filtration after refluxing hindered/

hindered filtration and gave high results and the reason for this is now obvious. High dilution followed by refluxing would keep the "amyloid" highly dispersed (perhaps colloidal) and able to pass through a filter, but delayed filtration and cooling would assist coagulation, hinder filtration and contaminate the lignin residue.

From the results with filtrate No.3 it seems that direct filtration through Whatman No.50 paper does not let the precipitate pass.

Experiments v and vi indicate that even after short times of strong acid contact, a humin precipitate is formed.

(vii) 0.5950 g. pretreated straw (corresponding to about 1 g. original straw) was treated with 25 ml 72% H_2SO_4 for 2 hours at 20°C and filtered through a Gooch crucible. 10 ml of the filtrate was diluted to 3% concentration, refluxed for 2 hrs and filtered through a sintered glass crucible (porosity 3). The filtrate was not clear so was boiled for ½ hour then allowed to settle, when a humin precipitate separated leaving some fine particles suspended. After refiltering through a sintered silica crucible (without filter aid) the liquid was still not clear so half of it was filtered through Whatman No.50 paper which retained some residue and gave a clear filtrate; the/

the other half was left for a day when a decided humin precipitate was obtained, which was dispersed into a homogeneous suspension on stirring. The suspension was filtered through a Gooch crucible or sintered silica crucible containing hyflo-supercel and the filtrate was clear and gave no precipitate on standing.

Thus the residue first precipitated appears to have a particle size intermediate between those of suspensions and colloids, and so may pass totally or partially through some filtering media. In lignin determinations when nitrogen or methoxyl are to be determined sintered glass crucibles are commonly used. So the variable fraction of the precipitate passing through will be both a contaminant and a source of variation in the lignin yield.

Portions of the original 72% acid filtrate were diluted 1½, 2, 3, 4, 5 and 7 times and allowed to settle for 6 hours. Dilution with 4 or more volumes of water gave a brownish precipitate and a clear supernatant liquid whilst the lower dilutions produced a yellow colour and turbidity, thus confirming that even low dilutions will not completely eliminate reprecipitation.

(viii) The previous experiment was repeated but a Gooch crucible was used for all filtrations and/

and the quantity of precipitate was determined.

Table 12 Data for Expt. viii

Wt. of pretreated straw sample = 0.60 g.(≡ 1g.original straw)

Vol. of 72% H_2SO_4 acid used =20.0 ml

Vol. of acid digest diluted = 6.0 ml

Wt. of ppte. (18.3940-18.3931g) = 0.9 mg

Wt. of total ppte. = $\frac{20}{6} \times 0.9 = 3$ mg.

∴ absolute error in lignin residue =

+ 3 mg per g. original straw.
= +0.3%

If the straw contains 10% lignin a result of 10.3% would be obtained, i.e. an error of 3% in the lignin content.

The data shown in Table 12 give an approximate indication of the magnitude of the precipitate from pretreated straw and it is seen that the quantity is small. With other feeding-stuffs rich in protein and carbohydrate and poor in lignin, the error would, however, be greater, so it is necessary to eliminate this source of error.

Determination of the amount of precipitate may serve to indicate the efficiency of a pretreatment.

(ix) To determine the influence of pretreatment on the yield of precipitate 0.5g. of original straw and 0.31 g. of pretreated straw were similarly treated with 20 ml H_2SO_4 for 2 hrs. at 20°C then filtered/

filtered through hyflo-supercel in sintered silica crucibles. Filtration was slow and incomplete but the filtrate obtained was very clear. When comparable quantities were diluted the precipitate from the straw sample was brownish white and much more bulky than from the pretreated straw which gave a brown coloured precipitate. In both cases the reaction to the cellulose test was negative.

(x) For comparison with 72% H_2SO_4 the behaviour of fuming HCl with straw was examined. It was possible to filter through hyflo-supercel in a sintered silica crucible, but a Gooch crucible was more satisfactory. Filtration was quicker than with 72% H_2SO_4 since the fuming HCl dispersed cellulose without becoming viscous. The results are shown in Table 13 (P 174).

With the pretreated straw, no humin precipitate was observed on diluting the filtrate, but on long standing (24 hours) the undiluted filtrate produced a precipitate. With the ethanol-benzene treated straw, a precipitate was observed on dilution of the fuming HCl filtrate after either 2 or 24 hours contact. (Treatments 5 and 7). The filtrate kept undiluted for 24 hours also produced a precipitate (Treatment 6.).

The main difference from reaction with 72% H_2SO_4/

H_2SO_4 is the production of a precipitate in the undiluted filtrate after 24 hrs; this was only observed with H_2SO_4 after a much longer period. This would be a source of error in the long contact period usually used with fuming HCl. With the original straw, the brown preicpitate was present in the diluted filtrate after either 2 or 24 hours contact and a precipitate was also produced in the undiluted filtrate.

Hence a short period of contact of the plant material with fuming HCl followed by filtration without dilution would eliminate both sources of error, but after a long period of contact filtration without dilution would lead to the retention of the "insoluble reversion products" in the lignin residue.

(xi) A comparison of 72% H_2SO_4 with fuming HCl in the treatment of seeds hay gave the results shown in Table 14 (P175). Precipitation on dilution occurred in all cases. In the case of seeds-hay (sample 2) fuming HCl gave a humin precipitate more bulky than that from H_2SO_4 whereas with pretreated straw (Expt.x) the filtrate after 24 hours contact gave no precipitate.

The humin residues were oven dried and then treated with 72% H_2SO_4 and fuming HCl. The acids were coloured yellowish brown but persistent residues/

residues remained, being suspended in the case of 72% H_2SO_4 but coming down in the case of fuming HCl. The behaviour of pretreated seeds hay with fuming HCl was different from pretreated straw but similar to the ethanol-benzene extracted straw. The pre-treatment of straw appears to remove all substances which can dissolve in fuming HCl and give a precipitate on dilution, but in the case of seeds-hay the pretreatment fails to do this.

3.2.6 Residues obtained from the First Filtrate after dilution of the strong Acid 7.5 Times.

After filtering an acid-plant mixture through a laboratory-made filter stick and dilution 7.5 times without boiling a slight brown humin precipitate was obtained on standing. It was filtered off through a sintered silica crucible with a filter aid, and found to dissolve in 72% H_2SO_4. This strong acid solution when diluted was almost clear, but it was not possible to determine whether or not reprecipitation occurred, owing to the very small amount of material. The residue is probably of the same nature as the precipitate obtained on diluting the strong acid digest (Section 2.5) but had passed through the filter.

Changes involved in the isolation of humin precipitates

(a) The precipitate was usually filtered through/

Table 13 Reaction of fuming HCl with straw (Expt. x)

Plant material	No.	Time of contact with fuming HCl (hrs.)	Time of retention of filtrate before dilution (hrs)	Vols of water added	Observations.
Pretreated straw	1	6	0	7.5	Clear white ppte. giving cellulose reaction, supernatant solution very clear.
" "	2	6	24 (in open tube)	7.5	Suspended white ppte. giving cellulose reaction.
" "	3	24	0	7.5	Very clear solution without any ppte.
" "	4	6	24 (in closed tube)	0	A suspended ppte formed in undiluted filtrate
Original straw, or straw only ethanol benzene extracted	5	2	0	2,3,4 and 7	Dirty folcculent ppte tinged brown and giving positive cellubse reaction.
do.	6	2	24 (in closed tube	0	Suspended ppte formed
do.	7	24	0	7.5	Ppte. giving negative cellulose reaction, leaving dirty solution.

Table 14 Comparison of 72% H_2SO_4 with fuming HCl in reaction with Seeds-hay (Expt. xi)

Plant Material	No.	Acid	Time of contact (hours)	Time of retention of filtrate before diln.	Vols of water added	Observations
Seeds-hay Sample 1	1	Fuming HCl	24 (room temp.)	0	7.5	Ppte giving negative cellulose reaction.
Ethanol-benzene extrd.	2	72% H_2SO_4	2½ (20°C)	0	7.5	As above.
Seeds-hay Sample 2 * pretreated	3	Fuming HCl	1 1/3 (room temp.)	24 (in closed vessel, room temp.)	10	Solution was turbid before dilution. A brown ppte. on diln resisting boiling for 2 hrs, ppte more bulky than in treatment 4.
	4	72% H_2SO_4	2 (at 20°C)	0	38	A brown ppte on diln., resisting boiling for 2 hrs.

* Pretreated according to method of Ellis et al.

through a small Whatman No. 50 filter paper and washed till free from chloride or sulphate according to the acid used. In one case when the filtrate gave a negative sulphate reaction the precipitate was oven dried. It blackened slightly, and when tested was found to be insoluble in 72% H_2SO_4, although it slightly coloured the acid. This behaviour was similar to that of a lignin residue prepared by the method of Ellis et al from Seeds-hay. In other cases the oven-dried precipitate was not changed in colour and almost completely redissolved in 72% H_2SO_4. When filtered through hyflo-supercel in a sintered silica crucible and then dried by suction the precipitate redissolved in 72% H_2SO_4 and reappeared on dilution, resisting refluxing.

It appears that testing the washings for freedom from sulphate may be inadequate in some cases and some H_2SO_4 may be retained in the residue itself producing partial carbonisation on drying and so changing its solubility. For this reason further extensive washing of the residue after a negative anion reaction in the filtrate is desirable. It seems possible that physical retension of H_2SO_4 by such humin precipitates was encountered by previous workers, (e.g. Hilpert and Littman's "Sugar Lignin") and the solubility of the residues so changed. When fructose/

fructose was treated with 72% H_2SO_4 then diluted 3 - 4 times and heated on a water bath (as in section 2.2 above) and the humin precipitate filtered and dried only by suction it almost completely redissolved in 72% H_2SO_4 and did not aquire the property of "lignin residue".

Conclusions

1. In the current methods of lignin determination involving dilution of the strong acid, formation of a humin precipitate resisting dilute acid hydrolysis is inevitable. Its amount is greater with unpre-treated plant material.

2. The only way to avoid this precipitation is to avoid dilution of the strong acid, and in the case of fuming hydrochloric acid, filtration should be after the short contact time necessary to disperse the cellulose.

3. Oven drying affects the nature of the humin precipitate, reducing its solubility in 72% H_2SO_4 and fuming HCl.

4. As the humin precipitate came down from 72% H_2SO_4 filtrates even after only slight dilution (to 1.1 vols i.e. 66% H_2SO_4), it appears that it may not be in true solution in the 72% H_2SO_4, but possibly in a highly dispersed and almost colloidal state.

5. The solubility in 72% H_2SO_4 of the freshly prepared precipitate/

precipitate indicates that it is not lignin and so makes necessary a quantitative examination of the determination of lignin without the dilution, which produces the humin precipitate.

6. This precipitate may possibly be a major source of error particularly with unpretreated materials. In addition to being a contaminant, its presence will affect the reproducibility of the final lignin residue.

3.2.7 The alcohol-soluble fraction of pretreated materials

In the "Reproducibility Study" with seeds-hay pretreated samples were washed with water to remove H_2SO_4, then dehydrated with alcohol and ether. Turbidity was observed in the alcohol washings and when heated, both large black and small brown particles separated leaving a turbid supernatent liquid. The amount of the residue which separated was appreciable. The properties of this alcohol-soluble fraction of pretreated materials were further studied in the following experiments.

Experiment (i) The results obtained in an examination of the combined washings from pretreated seeds hay are shown in Table 15.

Table 15 Examination of combined washings (containing water, alcohol and ether) from pretreated seeds hay

No.	Treatment	Observation
1	Some of the residue produced by heating the washings was filtered off and treated with 72% H_2SO_4	Most of the fine brown ppte. dissolved, but the large black particles resisted. The acid filtrate was clear and yellowish-brown.
2	The residue remaining undissolved in treatment No.1 was kept for 3 hrs in contact with 72% H_2SO_4	No dissolution.

51

Table 15 (cont.):

No.	Treatment	Observation
3a	The 72% H_2SO_4 solution from treatment No.1 was diluted 1½ and 7½ times.	A ppte with slightly brown colour came down.
b	Dilution to 3% H_2SO_4 and 2 hrs refluxing.	The ppte persisted, coagulating and leaving a clear supernatant liquid.
4	The ppte obtained on dilution in treatment No.3 was treated with 72% H_2SO_4 and the acid filtrate diluted, then refluxed.	Redissolution occurred in the H_2SO_4 giving a yellowish acid filtrate. Dilu. gave a clear solution and a whitish humin ppte (tinged brown) lighter in colour than that obtained in treatment 3.

It appears that the residue produced on heating contains at least two different components, one insoluble in 72% H_2SO_4 and the other soluble but easily reprecipitated by dilution. Repeated solution of the latter in 72% H_2SO_4 and precipitation by dilution gave a lighter coloured product resembling the precipitate obtained from the 72% H_2SO_4 filtrate of pretreated material.

Experiment (ii) This was carried out with a different sample of seeds hay pretreated according to the method of Ellis et al. The first alcohol washings were brown but washing was continued until the filtrate was only slightly coloured. The other washings obtained subsequently were quite clear. Treatments applied to the alcohol-water solution and results obtained are given in Table 16.

Table 16/

Table 16 Examination of alcohol washings of pretreated Seeds-hay (2)
(Expt. 11)

No.	Treatment	Observation
1	Alcohol filtrate (A.F.) heated	No precipitate
2	The alcohol filtrate was diluted with water and filtered partly through Whatman No.50 paper and partly through sintered silica crucible with filter aid.	Turbidity was produced on adding water. In both cases the filtrates were still turbid.
3	Alcohol filtrate heated with Fehlings soln.	No reduction.
4	1 ml Alcohol filtrate tested with a drop of alcoholic phloroglucinol reagent then conc. HCl added	No reaction with phloroglucinol alone but addition of HCl gave a coloration tinged with violet. On standing a coagulated ppte appeared.
4a	Treatment No.4 repeated substituting 60% alcohol for the phloroglucinol indicator (blank test)	No ppte. was produced, when conc. HCl was added but only a yellowish coloration.
4b	60% alcohol alone + conc. HCl (blank test)	Soln. clear and transparent.
5	A.F. treated with HCl solution of phloroglucinol	Turbidity first produced, then particles of ppte. appeared gradually. The mixture was tinged violet. On standing a flucculent ppte came down, (violet in colour but tinged with white and brown) leaving a clear, slightly yellowish-brown supernatant liquid.

Alcohol/

Table 16 (cont.)

No.	Treatment	Observation
6	Alcohol filtrate + dil. HCl	Turbidity first produced, followed fairly quickly by a flocculent brownish white ppte leaving a clear supernatant liquid and no violet coloration.
7	A.F. + H_2O, then dil. HCl	Turbid mixture first produced then addition of acid caused appearance of ppte as in No.6.
8	Ppte from No.6 and 7 filtered off, treated with alcohol, filtered.	Instant solution in alcohol giving clear brown filtrate.
9	Filtrate from 8 treated as 2 and 6	Gave same results as original A.F.
10	Filtrate from 8 treated with water and heated for 1½ hrs. in water bath	A brownish granular ppte appeared some floating and some sinking, leaving clear supernatant liquid.
11	Filtrate from 8 treated with HCl then heated on water bath 1½ hours.	Same results as 10, but supernatent still cloudy, with suspended particles of ppte.
12	Ppte from 6 washed with H_2O in a sintered silica crucible, then treated with 72% H_2SO_4	Ppte did not dissolve in H_2O, but dissolved fiarly quickly in 72% H_2SO_4 giving a clear brown solution.
13	The/	

Table 16 (cont.)

No.	Treatment	Observation
13	The 72% H_2SO_4 solution from (12) was diluted and heated 1 hr. on water bath	A brownish humin ppte. was produced and heating gave darker brown granulated particles as well as a brownish white flocculent ppte, and a clear supernatant liquid.
14	The brown ppte. after heating in 13 was filtered off, well washed, air dried, and treated with 72% H_2SO_4.	No solution occurred in 1½ hrs contact, the particles retaining their original shape, and the acid remaining uncoloured.
15	The strong acid filtrate from 12 was left 3 hrs without dilution.	Particles of ppte. came down.

The addition of water to the filtrate produces a turbid colloidal state in substances previously soluble in the alcohol.

The violet coloration in treatment 4 appears to result from a reaction between phloroglucinol and the material soluble in alcohol. Phloroglucinol in either alcohol or HCl solution gives the same ultimate result. The phloroglucinol reaction may indicate some relation to lignin, but on the other hand it may be due to traces of aldehyde.

Treatments 6 - 9 show that the alcohol-soluble material may be precipitated by dilute acid without affecting its ability to redissolve in alcohol and to be reprecipitated with acid.

Heating (treatments 10 and 11) granulates the precipitate produced by water or acid, but the granulated particles were found to be darker in colour than the original unheated precipitates so heating may involve some change in the nature of the precipitate.

Treatments 12 and 13 show that the substance dissolved in the alcohol washings is also soluble in 72% H_2SO_4 so that if it were left in the plant material it would be dissolved in the subsequent 72% H_2SO_4 treatment. It would, however, be reprecipitated on dilution and resist boiling so the precipitate obtained/

obtained in Section 2.5 above may contain substances of the same nature.

Treatment 14 shows that after solution in 72% H_2SO_4 and reprecipitation by dilution and heating there is a change in property leading to insolubility in strong acid. This change may occur to any part of the alcohol soluble fraction which is not completely removed from the pretreated material. Precipitation from 72% H_2SO_4 solution without dilution (treatment 15) is rather curious and suggests that the particles coming down were insoluble reversion products from the interaction of the dissolved "alcohol extractives" with the acid; alternatively the "alcohol extractives" may not be in true solution with 72% H_2SO_4 but merely dispersed and so ultimately granulating and precipitating. This behaviour is quite different from that of the precipitate obtained after the main treatment with 72% H_2SO_4.

Experiment iii. This experiment (Table 17) was similar to Experiment ii. The precipitate which dissolves in alcohol and 72% H_2SO_4 was tested for solubility in the same reagents after heating with 5% H_2SO_4.

The behaviour of the alcohol filtrate with dilute H_2SO_4 was the same as with HCl. From treatment No.5 it appears that dilution and heating produces/

Table 17 Examination of Alcohol washings of pretreated Seeds hay

Experiment (iii)

No.	Treatment	Observation
1	Alcohol filtrate treated with dil. H_2SO_4 and the ppte filtered off, washed with H_2O, and tested for solubility in alcohol and 72% H_2SO_4	Ppte instantly soluble in alcohol and almost completely soluble in 72% H_2SO_4.
2	72% H_2SO_4 solution of ppte diluted then heated 4 hrs. on water bath.	The ppte granulated on heating and turned dark brown leaving a not very clear supernatant soln.
3	Ppte from (2) filtered off and filtrate boiled in water bath.	A very clear filtrate obtained with no indication of any ppte.
4	Ppte from (3) tested for solubility in alcohol and 72% H_2SO_4.	Insoluble in both solvents.
5	Ppte from 1 washed with water then heated with 5% H_2SO_4 in boiling water and finally tested for solubility in alcohol and 72% H_2SO_4.	Ppte became dark brown and granular and insoluble in alcohol and 72% H_2SO_4.

Experiment (iv)

6	Residue from evaporated alcohol treated with (a) alcohol, (b) 72% H_2SO_4	Dissolved slowly in the cold alcohol more quickly on heating. Mostly dissolved in 72% H_2SO_4, but some particles remained insoluble even after 3 hours.
7	The residue from 6 was redissolved in alcohol, treated with H_2O, acidified with H_2SO_4, boiled; the granules formed were again tested (a) with alcohol (b) with 72% H_2SO_4.	Insoluble in both reagents.

produces complete separation of the precipitate, whilst treatments 4 and 5 indicate that boiling with dilute acid changes the nature of the precipitate. The change in treatment (5) is noteworthy as before extraction with alcohol the pretreated plant material had been refluxed with 5% H_2SO_4. It seems likely therefore that the 5% H_2SO_4 hydrolysis in the pretreatment may have rendered insoluble in alcohol and 72% H_2SO_4 some of the substances previously soluble in these reagents, so that alcohol extraction after this pretreatment process may only remove the unchanged portion of this fraction, the remainder leading to an increase in the lignin yield. Norman (1937 p. 172) suggested that 5% H_2SO_4 prehydrolysis may lead to the production of substances resistant to strong acid but this may not be such a rarity in view of the results obtained with two different hays.

Expt. IV

In/treatments 6 and 7 some of the alcohol washings were evaporated and the residue tested with results similar to those obtained with the original alcohol washings.

Discussion and conclusion.

The alcohol-soluble fraction of pretreated plant material is such that if it is not removed before the main treatment it will dissolve in the 72% H_2SO_4, but be reprecipitated on dilution and resist boiling; the/

the final acid hydrolysis will also ensure its complete insolubility leading to an increase in the lignin yield. There is a similarity in the properties of this fraction and that precipitated by dilution after the main 72% H_2SO_4 treatment, studied in Section 2.5 and it now seems that they may be merely two parts of the same fraction, that obtained on dilution after the main treatment representing the part which was not removed by alcohol after the pretreatment. To avoid errors due to this cause thorough extraction of the pretreated sample with alcohol prior to strong acid contact is essential and as in most 72% H_2SO_4 methods for lignin determination alcohol washing has been merely for dehydration, it will rarely have been adequate and so will have led to errors of variable magnitude. In methods (e.g. A.O.A.C. method) which do not involve washing with alcohol before acid contact this residue will be wholly recovered in the lignin residue.

It is now possible to account for the deviations between replicates in lingin determination by 72% H_2SO_4 methods using alcohol washing and in the previous reproducibility study when a homogeneous pretreated, alcohol-washed material was used the deviations were negligible. The satisfactory agreement between duplicates in the A.O.A.C. contact method (Phillips and/

and Goss 1936a) is probably due to the absence of any alcohol washing before the strong acid contact, and to the use of a homogeneous pretreated sample.

Avoiding dilution of the strong acid digest would also eliminate the error from this source.

It appears that during 5% H_2SO_4 hydrolysis in the pretreatment a change occurs producing a substance soluble in alcohol and 72% H_2SO_4 but prolonged hydrolysis gradually renders it insoluble in these reagents and insoluble material produced as an artefact in the 5% H_2SO_4 treatment would not be removed from the lignin residue by avoiding dilution of the strong acid digest.

3. 2.8 Comparison of the precipitate obtained by diluting strong acid digests with that obtained from the alcohol-soluble fraction of pretreated material with alcohol.

(a) A sample of pretreated straw was treated with 72% H_2SO_4 for 3 hours at 20°C filtered, and the acid diluted to 3%. The precipitate obtained was filtered off and readily dissolved in alcohol. The alcoholic solution was treated with dilute H_2SO_4, and then boiled; turbidity was first produced and then a precipitate formed exactly as occurred withthe alcohol-soluble fraction from pretreated materials.

(b) A sample of pretreated seeds-hay was digested with 72% H_2SO_4 for 2 hours at 20°C and the acid/

acid digest when treated as in (a), produced a humin precipitate which dissolved in alcohol. With dilute acid this solution became turbid and when left overnight a humin precipitate separated. This was filtered in a sintered silica crucible and treated with water, which leached out the substances as a turbid colloidal filtrate.

When treated with dilute H_2SO_4 and left overnight the brown humin precipitate reappeared.

It would seem that the precipitate obtained from the dilution of strong acid digests and that obtained from the alcohol-soluble fraction of pretreated plant material are identical, although by interaction with soluble associates strong acid contact may produce additional material of the same nature.

In the current methods of lignin determination dilution of the acid digest and immediate filtration followed by thorough washing with water may remove this contaminating fraction in colloidal form but the porosity of the filtering medium and the time taken for filtration (which may change the size of the dispersed particles by granulation) will affect the extent to which this is achieved. It was noted in the previous reproducibility study that where a filter stick was used for filtering off the freshly diluted 72% H_2SO_4-plant mixture the humin precipitate was/

was observed in the filtrate.

3.2.9 The alcohol soluble fraction of plant material extracted with ethanol benzene and digested with acid pepsin.

A 20 g. sample of seeds-hay was extracted with ethanol benzene and digested with acid pepsin as in the method of Ellis et al, then washed free from HCl, dehydrated with alcohol and ether, and dried below 50°C. The alcohol washings were only very slightly tinged brown and evaporation gave a brown residue the amount of which was very much less than that described in Section 2.6.

The residue mostly resisted solution in alcohol but boiling for a while produced a slight coloration inthe alchhol.

The residue dissolved readily in 72% H_2SO_4 giving a clear solution.

Diluting this acid solution 7.5 times gave a precipitate which resisted boiling. The precipitate was at first flocculent white or light brown in colour but on boiling turned brown and coagulated leaving a clear supernatant liquid.

A portion of the 72% H_2SO_4 solution was kept undiluted for 24 hours at room temperature and no precipitate was observed although dilution then gave a precipitate with the same properties as before.

Thus evaporating the alcohol solution affects the/

the alcohol solubility of the residue (cf. with evaporating the alcohol washings of a completely pretreated sample) but solubility in 72% H_2SO_4 is similar to that of the residue from a completely pretreated sample, and to that of the precipitate obtained by diluting strong acid digests.

3.2.10 Residue obtained from 5% H_2SO_4 filtrate in pretreatment.

Many workers have recorded humin residues separating from dilute acid hydrolysates in the pretreatment, and some workers have considered it to be lignin. The 5% H_2SO_4 filtrate from seeds-hay was examined in the following experiments.

Experiment i. The results of this experiment (Table 18) indicate that the residue dissolves in strong acid and is reprecipitated by dilution, but after 2 hours contact with the strong acid no precipitation occurred on dilution so it appears the precipitate may undergo hydrolysis under these conditions.

Experiment ii This was carried out with a different sample of hay (Table 19). The residue was almost insoluble in alcohol and oven drying did not affect its solubility in strong acid. Long contact with strong acid did not produce any insoluble reversion substances as was found with the alcohol-soluble residue/

(Continued on p. 197)

TABLE 18.

Examination of residue from 5% H_2SO_4 filtrate of Seeds-hay, Sample 1 (Expt. i)

No.	Treatment	Observation
1.	Filtrate stirred and filtered through sint. silica crucible with filter aid.	ppte remained, and filtrate clear.
2.	Ppte in 1 treated with 72% H_2SO_4 and filtered.	soluble, giving clear brown solution
3.	72% H_2SO_4 soln. from (2) diluted	Turbidity, followed by precipitation.
4.	Original 5% filtrate, filtered through small Whatman No.50 paper disc, half the residue treated with 72% H_2SO_4, the other with fuming HCl	Residue soluble in both reagents.
5.	The two acid solutions from 4 diluted after two hours.	No ppte. observed.

TABLE 19

Examination of residue from 5% H_2SO_4 filtrate of Seeds-hay, Sample 2 (Expt.ii).

No.	Treatment	Observation
1.	A portion of the filtrate filtered through sint. silica crucible with filter-aid, washed with water.	ppte. did not dissolve in water.
2.	The ppte. in (1) treated with alcohol.	Did not dissolve. The alcohol residue clear.
3.	The ppte. in (1) treated with 72% H_2SO_4.	dissolved instantly, giving clear brown solution.
4.	The ppte. in (1) was washed, oven dried and treated with 72% H_2SO_4	Solution in the acid was slower than in (3)
5.	The ppte. in (1) was digested on water bath for several hours with 5% H_2SO_4.	No observable change in appearance of ppte.
6.	The solution from (3) was left for four days.	A change in colour occurred, but no ppte. formed.
7.	The acid solution from (3) was diluted immediately.	A brownish humin ppte. formed which resisted heating for 3 hrs. on water bath.
8.	The ppte. from (7) was filtered off and treated with 72% H_2SO_4.	Some dissolved but most remained insoluble.

9./

TABLE 19 (Cont.)

No.	Treatment	Observation
9.	The strong acid solution from (8) was diluted the next day.	Solution appeared clear at first, but on standing, turbidity appeared. Heating on a water bath gave a ppte. much lighter in colour than in (7).
10.	The ppte. obtained in (7) was dissolved in 72% H_2SO_4 and the solution diluted.	PPte. obtained, very light in colour.
11.	The acid solution from 3 was diluted on 2nd day.	No ppte. occurred, the solution remained clear after 4 hours.
12.	The diluted solution from (11) was heated in water bath for 3 hours.	Ppte. appeared at first light brown, heating gradually turning it darker brown.
13.	Ppte. from (12) treated with 72% H_2SO_4.	Some dissolved but mostly insoluble.
14.	Treatment (2) repeated with a large amount of the residue.	Slight amounts of residue dissolved in the alcohol and later came down slightly brown.
15.	Some of the residue from (1) examined with alcoholic phloroglucinol reagent and conc. HCl.	No violet or reddish colouration produced, whether the residue was filtered through paper or through silica crucible.

* Table 19 for experiment 11, see/

residue obtained in the washing of pretreated material (p.179) Perhaps its amount was small.

The residue is different in nature from other residues, and from lignin. Although prolonged heating on a water bath, turned most of it into granules insoluble in 72% H_2SO_4, some still remained soluble (Treatment No.8).

The acid solution of the residue when left for a period then diluted, did not produce reprecipitation ~~but~~ until heated in a water bath (11, 12).

2.11 Residue from the 72% H_2SO_4 filtrate of Ethanol-Benzene extracted seeds hay

Seeds hay after extraction only with ethanol benzene was treated with 72% H_2SO_4 for 2 hours at 20°C and then filtered giving a very clear filtrate. When left to stand for a few days a bulky brown humin precipitate settled down leaving a very clear supernatant liquid.

It was concluded that long contact had produced insoluble reversion products. The filtrate and residue were examined as shown in Table 20.

The results indicate that the residue is a mixture of at least two different components, one soluble in both 72% Acid and alcohol, the other soluble only in 72% H_2SO_4.

The former resembled the residue obtained from the/

Table 20 Examination of residue from 72% H_2SO_4 filtrate of ethanol benzene extracted seeds hay

No.	Treatment	Observation
1	Supernatant liquid diluted	No. ppte. occurred, the solution remaining clear.
2	Residue filtered off washed with H_2O, treated with alcohol.	A portion dissolved in the alcohol giving it a brown colour.
3	The alcohol solution from (2) was treated with 5% H_2SO_4	Turbidity and precipitation occurred.
4	The alcohol-insoluble residue from (2) was treated again with alcohol	The alcohol remained clear, and gave no turbidity with 5% H_2SO_4. The residue remained insoluble.
5	The insoluble residue from (4) was treated with 72% H_2SO_4	Dissolved instantly, giving very clear solution.
6	The strong acid solution from (5) was diluted	slight turbidity.
7	The residue from (2) treated with 72% H_2SO_4	Dissolved instantly giving clear soln.
8	The strong acid solution from (7) was diluted	A ppte. formed which resisted heat.
9	The residue from (2) was thoroughly washed and dried at 100° then treated with (a)72% H_2SO_4, (b) alcohol	Dissolved slowly in 72% H_2SO_4 but almost insoluble in alcohol.
10	The alcohol filtrate from (9) was treated with 5% H_2SO_4.	No turbidity or ppte produced.

the alcohol washings of a pretreated hay.

The residue as a whole is soluble in 72% H_2SO_4 reprecipitated by dilution, and resistant to boiling with dilute acids. Although the residue originally separated from strong acid solution after filtering and washing, it unexpectedly redissolved in the same acid. This may indicate that the residue is not in true solution in the acid but perhaps highly dispersed and analogous in behaviour to the residue obtained from alcohol washings of pretreated hay (see Section 2.7 Expt. 11) P185.

2.12 Summary and conclusions (Section 2)

A study has been made of the behaviour of strong acid with plant material at low temperature and with pure substances, and plant material under the conditions of lignin determination. Residues obtained at various stages during lignin determination have been studied mostly in relation to their physical properties, solubility in strong acid and alcohol, and behaviour towards dilute acid hydrolysis. The significance of these residues as sources of errors in lignin determination has been examined. Comparisons have also been made of different acid reagents used in lignin determination using pure cellulose to study their relative efficiencies to disperse and hydrolyse cellulose and to choose the best workable reagent.

Hilpert/

Hilpert and Littman's view that lignin is an artefact has not been confirmed.

The formation of a humin precipitate on diluting strong acid hydrolysates of plant material occurred even at 0°C, although the amount at low temperature was very small. This residue is formed through interaction of the strong acid with some associates or during the pretreatment ~~or~~ or it may be present originally in the plant itself. There was experimental evidence indicating its formation during pretreatment.

The time of digestion with strong acid is best determined by applying a cellulose test to a small drop of the mixture. Two hours at 20°C with 72% H_2SO_4 is adequate to prevent reprecipitation of cellulose on dilution and it is advisable to redigest (with fresh 72% H_2SO_4) any plant particles sticking to the sides of the container until a negative cellulose reaction is obtained.

There is a similarity between the various humin residues obtained from the alcohol washings after pepsin-HCl digestion, or after dilute acid prehydrolysis, and from dilution of 72% H_2SO_4 digests of plant material; residues separating from the filtrate of dilute acids used in prehydrolysis or from the filtrate after dilution of 72% H_2SO_4 digests are/

are also similar; they all dissolve in 72% H_2SO_4, are reprecitated by dilution, resist dilute acid hydrolysis, and exhibit colloidal properties.

The possibility indicated by the reproducibility study (Section 1) that some analytical error occurs between the dilute acid prehydrolysis and the main and final treatments has been confirmed. The formation of insoluble residues by 5% H_2SO_4 prehydrolysis is substantiated, and is believed not to be exceptional.

On the assumption that lignin is insoluble in strong acids, avoiding the dilution of strong acids in lignin determination is necessary to correct a serious error in the current methods. Quantitative work is needed to study this modification and its effect on lignin yield and qualities.

A distinction between true solubility in 72% H_2SO_4 and dispersion may be necessary to decide whether a substance is a lignin or not. Perhaps lignin does not dissolve in 72% H_2SO_4 but can be highly dispersed in it. The humin residues obtained during lignin determinations and considered meantime as contaminants, may not be truly soluble in 72% H_2SO_4 and may have some relation to lignin. Further study of the qualities of these humin residues (other than solubility) and their analytical constants is needed to determine their relation to lignin.

3.5. Colour Reactions for the identification of Lignin

A study was made of the reliability of certain colour reactions and attempts made to overcome some difficulties in performing them; the reaction in the plant tissue was compared with that in prepared lignins .

3.5.1 The Phloroglucinol test

Straw or seeds hay samples either untreated or after pretreatment were used. Acid and alkali lignins were also used.

(i) In the tissues the colour developed whether the phloroglucinol was in HCl or alcohol solution, but some plant particles did not respond to the test whereas the majority were coloured.

(ii) In acid lignin it was not possible to identify the colour; it was either obscured by the dark colour of the lignin after 72% H_2SO_4 treatment, or the 72% H_2SO_4 had removed the traces of materials responsible for the colour.

(iii) Pretreated plant material treated with ethyl aceto-acetate (after moistening with conc. HCl) gave a plant residue which did not respond to the phloroglucinol reaction although it produced an acid lignin with 72% H_2SO_4. The solvent therefore removed something which reacts with phloroglucinol, although lignin material remained.

(iv)/

(iv) The colour developed by plant tissues with phloroglucinol was found to be removed by alcohol and ether washing. The test was repeated on the washed material, and the colour reproduced.

(v) Recently Russel (1948) demonstrated that alkali lignin and a synthesized lignin model when dispersed on filter paper gave the characteristic colour reaction with phloroglucinol in 12% HCl.

The reaction was tested with alkali-lignin, prepared from seeds-hay using pure ashless filter paper. The colour developed gradually as the filter paper dried. A control test was made moistening the filter paper with the reagent and without the alkali lignin, and the colour developed, in the same way and with the same intensity as when alkali lignin was used. It was thought that the filter paper possibly contained traces of lignin or aldehydes which could produce the colour, but when a layer of hyflo-supercel (previously ignited at 800°C) was moistened with the reagent the colour developed in the same way. These tests were repeated with A.R. phloroglucinol and the same results obtained.

It was concluded that the test is not reliable, in agreement with Harlow (1927) 1928. who found that the colour with phloroglucinol varied considerably, and that too great reliance on it led to error (Dorrée, 1947, p.339)

3.3.2 Maüle test

This widely used test was found to succeed in plant tissues but in the case of acid lignin, the colour was not obtained. It was found to be unsuitable for alkali lignin which dissolves in the ammonia used (after treatment of the material with 1% $KMnO_4$).

The colour is unsuitable as a general test for all forms of lignin.

3.3.3 Chlorine-Sodium sulphite test.

This test was performed by the addition of sodium hypochlorite solution to the plant tissue or lignin in a test tube then a few drops of dilute HCl or H_2SO_4 were added, the supernatant solution decanted, the residue washed once with H_2O and Na sulphite solution added; a pink to purple-red colour developed in the solution.

The colour developed in both plant tissues and prepared lignin. In some cases where the colour failed to appear, it was found that the reason was the extent of the washing prior to the addition of sodium sulphite solution. If H_2O washing was omitted the colour usually did not appear, due to the presence of excess chlorine as found by Campbell and McOowan (1939). Excessive washing also prevented the development of the colour, probably due to removal

of/

of the soluble chlorolignins.

After decanting the acidified Na hypochlorite solution one washing with a small amount of H_2O was adequate to remove the excess of chlorine and leave a sufficient amount of chlorolignin to produce the colour.

In the cases of alkali lignin, the colour development was not obtained or the colour was brown. It was observed that the alkali lignin was easily chlorinated and was mostly dissolved and lost during the decanting and washing with water. Moreover when the alkaline sulphite solution was added, the remaining lignin dissolved producing a brown colour which masked the colour reaction.

It was found in a study of alkali lignin (p252 text) that this was largely insoluble in 72% H_2SO_4 and the treatment with acid led to greatly reduced solubility in alkali.

These properties were used to modify the test to suit alkali lignin; the alkali lignin was treated with 72% H_2SO_4, then this was diluted with water, mostly decanted and sodium hypochlorite solution added. After again decanting and washing once with H_2O, and Na_2SO_3 solution was added and the colour produced. This procedure was adopted to test dried humin residues obtained during lignin determination.

It/

It was concluded that this test was more reliable than the phloroglucinol or Maüle tests for lignin whether 'in situ' or isolated.

3.3.4. Phenol Reagent

This reagent is specific for reactive aromatic hydroxy groups, but the colour is also given by the indole group of tryptophane. In the absence of interfering aromatic amino acids the test may be useful for detecting lignin and even with lignin residues containing nitrogen if the amount is small the interference is negligible, since the tyrosine and tryptophane in plant proteins together amount to less than 6%. (Sahyun, 1944, p.67) Used in conjunction with the ninhydrin reaction the test was found useful for acid lignin hydrolysates. In some cases the ninhydrin reaction was negative and the phenol reaction positive, the phenolic groups, probably being from lignin dissolved by the hydrolysis. However there are limitations due to differences in the sensitivity of reagents.

Alkali and ester lignin preparations (Chapters IV & VI) responded well to the test and in several cases these preparations were almost free from nitrogen. This led to the conclusions that the presence of reactive phenolic groups in lignin is most probable as claimed by several workers. (e.g. Brauns formula -

P 30/

p 30, XLI appendix) but disputed by Freudenberg whose formula for lignin (p.30/ XXXIII appendix) contains no free phenolic group.

3.4. The determination of lignin without dilution of the strong acid digest.

A serious error was discovered in the current procedures for lignin determination, and it was concluded that avoidance of dilution of the strong acid in the main treatment might correct this error and shorten the procedure. This was tested in a preliminary experiment and then search was made for a more practicable filtration procedure, using several different strong acids and acid mixtures, and differently pretreated plant materials.

3.4.1 Preliminary experiments

(1) Experimental work. The plant material was seeds-hay pretreated according to the modified procedure of Ellis et al (Appendix p.17). A large sample was pretreated washed with H_2O and then with alcohol until the washings were colourless (in order to remove the alcohol-soluble material) and finally with ether; after air drying or warming at low temperature a portion of the homogeneous sample representing 1 gram of original material was used for further strong acid treatments. Fuming HCl was used to dissolve associates and disperse cellulose because the/

the filtration of fuming HCl containing freshly dispersed cellulose (45 - 50 mins.) was easier than that of 72% H_2SO_4 containing dispersed and hydrolysed cellulose (2 hrs.) Gooch crucibles with asbestos mats were quicker than sintered silica or glass crucibles. Digestion of the plant material in fuming HCl was carried out in well stoppered, wide mouthed glass containers, and it was observed that so long as some of the plant particles remained sticking to the sides of the container the dispersion of cellulose was incomplete and the mixture viscous, but if the particles (lignin residue) moved freely, it was a sign of dispersion and of the readiness of the mixture for filtration. The digestion took about 1 hour at room temperature.

As freshly dispersed cellulosic material was reprecipitable by dilution or by decrease of HCl concentration, it was decided to test the adequacy of the fuming HCl in removing associates. This was done by further treating the lignin residue after washing and drying with 72% H_2SO_4. An adequate amount of the acid was added to the residue in the Gooch crucible and left in contact with it for 2 hrs. with occasional stirring (this was possible as a perforated porcelain disc was used over the asbestos mat in the Gooch). 2 hours were considered adequate because it was found that dried reprecipitated cellulose (dispersed/

(dispersed in fuming HCl and precipitated by dilution) dissolved in 72% H_2SO_4 much more quickly than the original cellulose.

After treatment with both fuming HCl and 72% H_2SO_4 as described above, the dry lignin residue was boiled for 1 hour with 3% H_2SO_4. (By placing the Gooch crucible with the residue in a sintered silica crucible, and immersing both in the boiling acid so that the Gooch crucible was not submerged but the lignin residue was covered by the hot solution.)

After treatment with fuming HCl, the residue was washed with fuming HCl until the filtrate (neutralised with NaOH) showed no reduction of Fehling's solution. (The first 3 or 4 washings exhibited slight reduction). After 72% H_2SO_4 contact washing with 72% H_2SO_4 was continued also until no reduction of Fehling's solution occurred.

The filtration of 72% H_2SO_4 at this stage was quicker than that of the fuming HCl in the first stage and much quicker than the filtration of 72% H_2SO_4 when used alone for hydrolysis. In one case the 72% H_2SO_4 filtrate was tinged brown indicating that this acid could remove something not extracted by the fuming HCl.

After washing with strong acids, water, alcohol and ether, the residue was dried at 105°C . The aim of/

of washing with alcohol was to see if this would dissolve anything from the residues after treatment with strong acids, but no coloured washings were obtained.

The I_2-H_2SO_4 reaction for cellulose was negative in the final residues.

(ii) Standard of comparison Plant material similarly pretreated was digested with 72% H_2SO_4 for 2½ hours at room temperature (below 20°C). The particles sticking to the digestion container, were treated with fresh 72% H_2SO_4 for a further ½ hr. then diluted 7.5 times, filtered, and finally refluxed 2 hrs. with 3% H_2SO_4. (A modified Ellis et al procedure.)

(iii) Results and discussion The results (Table A7) indicate that by avoiding dilution of the strong acid both the lignin residue and the ash-free lignin are reduced. Even using only fuming HCl for 80 minutes removes associates more efficently than the treatment of the modified current method.

The standard 72% H_2SO_4 procedure gave yields 8% greater than the use of fuming HCl alone and this is probably a significant difference for replicates starting from the same homogeneous pretreated material. This was expected since reprecipitation of the humin residues soluble in the strong acids was avoided/

avoided.

That 72% H_2SO_4 can remove something not extracted by fuming HCl is shown by sample 2 in Expt. 1 where there was 2.6 mg reduction (on basis of 1g. original sample) following treatment with 72% H_2SO_4. This treatment after the reaction with fuming HCl appears to be necessary. The final hydrolysis in the tested procedure may lower the yield of ash-free lignin, but the difference may not be significant.

The ash content of the final residue was slightly higher in the test procedure (14.9%) than in the standard procedure (15.0%) but the absolute ash contents were very similar (1.1% and 1.0% of original sample). Possibly the absolute ash content of the lignin is not affected by the final dilute acid hydrolysis.

Although the filtration of strong acid without dilution (see Table A 11 p.24) was reported by Schwalbe and Becker (1919) and Heuser and Skioldebrand (1919) using fuming HCl, and by Popoff (1938) using HCl-$ZnCl_2$ mixture, such procedure has not been reported for 72% H_2SO_4, and no emphasis has been made of the necessity for avoiding dilution.

3.4.2 Determinations using various pretreatments and strong acid digestions.

The same plant material was used as in section

4.1/

4.1 but three samples were pretreated differently viz (1) only extracted with ethanol-benzene, (2) ethanol-benzene extracted and pepsin-HCl digested, (3) ethanol-benzene extracted and trypsin -Na_2CO_3 digested. Another sample was pretreated, according to the modified method of Armitage et al. (Appx. PP. 15-17) After pretreatment the samples were finally washed with alcohol and ether, then dried by suction, or in an electric oven at 45° to 50°C. 10 to 20 g. samples were pretreated at one time, and weights corresponding to 1 g. of the original material taken for analysis. In table A8 the yields and composition of the pretreated residues are shown. These residues were the starting material for the subsequent treatments with various strong acids and so are designated starting material No.1 (S.M.1) etc. in Table A8 and in the following Tables A9 to A13, which indicate the main treatments used to remove the associates of lignin from the starting materials and the results obtained. Most of the results are expressed on an air-dry basis, but as ~~is realised~~ slight changes in dry matter content occurred, the later results are expressed on the more exact dry matter basis. The standard procedure used for comparison was that of Ellis et al (1946) modified as described in Section 4.1(ii). The modified procedure of Armitage et al (1948) was also/

also examined omitting the prehydrolysis with 5% HCl and avoiding dilution of the strong acid.

Experiment (i)

This was carried out on similar lines to the preliminary experiments (Section 4.1) and the results are shown in Table A9.

Treatment No. 1 was the modified procedure of Ellis et al as used previously. In treatment No.2 fuming HCl was used for 80 mins followed by filtering, then 72% H_2SO_4 digestion for 2 hours in the Gooch crucible then filtering and washing with 72% H_2SO_4 and then water. The filtration of the fuming HCl was much easier when the plant material was fully pretreated (S.M.4) than when the 5% H_2SO_4 prehydrolysis was omitted (S.M.3) or both this and the pepsin digestion omitted (S.M.2). The time of filtration was variable, thus making the actual time of contact with fuming HCl variable; manipulation was unpleasant.

In some cases (with S.M. 2 and 3) filtration ceased and the determinations had to be abandoned. Treatment No.3 employed the proposed mixture (1) instead of fuming HCl, digesting for 2 hrs because the dispersing power was less than that of fuming HCl; the HCl fumes from this reagent were less, but filtration difficulties remained. In treatments

4/

4 and 5 the proposed mixture (2) was used and in treatment 5 the final washing was with boiling 3% H_2SO_4 which was considered to be more efficient than water in removing carbohydrates but not so severe as the final hydrolysis commonly employed. This proposed mixture (2) appeared to be less liable to produce undesirable reactions than the others and did not darken in contact with plant material. It had the advantage of being stable and easy to prepare and produced less HCl fumes than the other reagents but after 2 hrs reaction, filtration was more difficult than with ~~the mixture in 2 hours was more difficult than with~~ fuming HCl or the proposed mixture (1). It was found that when plant material was treated with 72% H_2SO_4 and filtered without dilution (through glass wool) then 1 volume conc. HCl added to the filtrate (producing the proposed mixture No. 2) turbidity and slight precipitation occurred, indicating that the 72% H_2SO_4 was more efficient than the proposed mixture 2 for dissolving associates.

Results: The ash-free lignin obtained from the fully pretreated material (SM4) was less in treatments 2 and 3 (7.30 and 7.41% of original DM) than treatment 1 (7.86%). The results for fully pretreated material were lower than for plant material only ethanol benzene extracted (SM2) but, somewhat surprisingly, they were higher than when the H_2SO_4 hydrolysis was

omitted/

omitted from the pretreatment. Thus the 5% H_2SO_4 prehydrolysis increased the yield of ash-free lignin although treatment with 5% H_2SO_4 removes associates (vide Table A8) and would therefore be expected to reduce the errors arising from condensation of associated with lignin. Since this acid prehydrolysis may remove some of the lignin fraction (Harris & Mitchell, 1939, Cohen and Harris, 1937, Gupta, 1948) it is concluded that the increase in ash-free lignin which it produced is due to the formation of material insoluble in 72% H_2SO_4. Previous experiments (section 2 P185 text) with the alcohol-soluble residue after dilute acid hydrolysis showed that heating with dilute acid granulated it and rendered it insoluble in 72% H_2SO_4, and it was concluded that this change could also occur in the plant material during pretreatment with dilute acid.

The production of the highest yield of ash-free lignin from plant material only extracted with ethanol benzene ~~extracted~~ must be due to the presence of substances removed by the pepsin-HCl treatment and which can condense with lignin in the 72% H_2SO_4 treatment. Carbohydrates are unlikely to increase lignin yield by the formation of insoluble material with 72% H_2SO_4 (see Section 2, P 147) and whether proteins will do so is uncertain. The work with protein reported in section 2 (P143) was limited to gelatine, and the earlier/

earlier work of Phillips,(1939) and Norman,(1937) was carried out by diluting the strong acids and observing the formation of small or negligible insoluble residues. Condensation with lignin may be the major cause of contamination.

Experiment (ii)

It was observed that conc. HCl alone did not change the structure of plant particles as it did with pure cellulose, but with the addition of a suitable amount of 72% H_2SO_4 the mixture could disintegrate plant cellulose material forming a viscous pulpy mixture. It was thought that if plant material could first be disintegrated with the minimum volume of that mixture and then a relatively large amount of 72% H_2SO_4 added, (giving a final acid mixture with only slightly less than 72% H_2SO_4 and a small amount of HCl) it might be possible to shorten the treatment by avoiding two successive acid digestions. In treatment No. 6 used in this experiment plant material equivalent to 1g. of the original (hay) was treated with 1 ml. conc. HCl followed by 4 ml. 72% H_2SO_4 for 15 minutes, stirring the mixture, then 30 ml. 72% H_2SO_4 were added and reaction continued for 2½ hours, after which the residue was filtered off, washed with 72% H_2SO_4 and then water. With SM2 and SM3 washing was first with 72% H_2SO_4 and then with conc. HCl. The results (Table A9) confirm

those/

those of Experiment (i), SM2 giving the higher figure and SM3 the lower. The difference between the starting material fully pretreated (SM4) and that with the omission of 5% H_2SO_4 prehydrolysis (SM3) was, however, small.

Filtration was still slow, and foaming occurred during mixing of the acids and stirring; the foaming was overcome by shaking in a stoppered bottle instead of stirring, but care was needed when opening the bottle. In the final washing with 72% H_2SO_4, the acid only percolated slowly in the case of the plant material not fully pretreated, so washing with conc. HCl, which percolated more quickly, was substituted.

Experiment (iii).

In treatment 7 (Table A9) 4 ml. conc. HCl and 2 ml. 72% H_2SO_4 were used to disintegrate the cellulose, then 50 ml. 72% H_2SO_4 were added for 2½ hours, followed by filtering and washing with 72% H_2SO_4. Treatment 8 was the same except that the acid mixture was diluted, before filtering and washing with 72% H_2SO_4 (to overcome the difficulties in filtering the strong acid); after further washing with water, the humin precipitate could be removed from the residue by washing with 72% H_2SO_4, and then hot 3% H_2SO_4. Filtration was still slow, however, and/

and it seems that the solution of the humin residue in 72% H_2SO_4 was the cause. Treatment 9 was similar to treatment 6 of experiment (ii), but the mixture was mechanically shaken in a stoppered bottle, whilst treatment 10 was conducted on similar lines to treatment 4 of Experiment (i) using the proposed mixture (2) and redigesting the residue with 72% H_2SO_4 in a beaker instead of a Gooch crucible (to allow better contact and stirring). After treatment with the HCl : H_2SO_4 mixture the plant residue formed a mass which did not break into small particles in the 72% H_2SO_4 and filtration was difficult.

In this experiment the ash-free lignin from plant material treated with ethanol-benzene and pepsin-HCl was less with treatments 7 and 8 than in Experiments (i) and (ii), but with treatments 9 and 10, which were difficult to carry out, the results were higher than in Experiments (i) and (ii). Lumping and slow filtration apparently lead to contamination of the lignin residue. In treatment 10, SM2 gave an ash-free lignin with a much higher nitrogen content than SM3; the figures for corrected lignin from SM2 and SM3 were quite close and lower than the corrected lignin obtained by the standard procedure.

Experiment (iv)./

Experiment (iv).

The previous results showed that after ethanol-benzene extraction (SM2) further treatment with pepsin (SM3) lowered the insoluble lignin residue in 72% H_2SO_4. It was thought desirable to replace the pepsin-HCl digestion with a purely chemical treatment if possible and treatment with fairly strong, cold acid was tried. A sample of ethanol-benzene extracted material was soaked for 6 hours in 20.24% HCl at room temperature, then filtered and washed with conc. HCl, water, alcohol and ether. The final residue was 42% of the original dry matter (the comparable figures for ethanol-benzene residue and fully pretreated residue being 87.5% and 38% respectively) indicating that the treatment is nearly as efficient as the combined pepsin digestion and 5% H_2SO_4 hydrolysis. The residue was treated directly with 72% H_2SO_4, and it was observed with surprise that after about 2 hours the particles of plant material were all suspended in the mixture, and none were sticking to the sides of the container or stirring rod; after 3 hours contact filtration of the mixture directly through a Gooch crucible was rapid. The experiment was repeated (Treatment 12) using conc. HCl and soaking for 2 hours and the absence of particles sticking to the vessel and

rapid/

rapid filtration were again observed. These pretreatments appeared to be preferable to any of the others used previously as they were effective in removing associates yet gave mixtures with desirable physical properties. The filtration of the conc. HCl mixture was best achieved by using a Gooch crucible fitted with a disc of Whatman No.50 filter paper; the washed and dried residue was easily transferred quantitatively for digestion with 72% H_2SO_4, but it was also possible to digest the plant residue (along with the filter paper disc) in the Gooch crucible itself by placing the crucible in a suitable beaker. The filter paper disc was observed to be disintegrated and dispersed much more quickly than a disc not treated with conc. HCl. The result obtained from treatment No.11 indicated that the acid treatment could not satisfactorily replace digestion with pepsin so treatment (12) was applied to plant material which had been treated with both ethanol-benzene and pepsin-HCl. The residue after treatment with conc. HCl was 32.8% of the original dry matter, which is less than the residue obtained by the full pretreatment used for SM3. (38.5%). Thus it appears that the conc. HCl extraction may very well replace the 5% H_2SO_4 prehydrolysis, besides rendering/

rendering practicable the filtration of 72% H_2SO_4 without dilution.

The ash-free lignin obtained was of the same order as before (excluding the high result obtained with treatments 9 and 10), with the exception of treatment (7) (Experiment (iii)) which was the lowest of all. Pepsin digestion appears to be necessary to remove something which conc. HCl failed to remove, undoubtedly proteins. This resulted in a higher ash-free lignin from samples only treated with ethanol-benzene than from those treated also with pepsin-HCl.

The nitrogen data obtained in treatment 10 (Experiment (iii)) showed that when no pepsin treatment was employed the ash-free lignin contained a much higher nitrogen percentage but, correction for N brought the residues from SM2 and SM3 much closer together.

Experiment (v).

Treatment 13 (Table A 11) used in this experiment was similar to treatment 12, the time of contact with conc. HCl being shortened to avoid possibly unfavourable changes in soluble associates. The starting material was treated for 15 minutes with conc. HCl, then filtered, washed (with conc. HCl till the washings were clear) and dried. It was then treated/

treated for 3 hours with 72% H_2SO_4, filtered, washed with 72% H_2SO_4 until the washings were clear, and finally washed with water.

The results (Table A 11) for ash-free lignin are in favour of this treatment. The samples pretreated with both ethanol-benzene and pepsin (SM3) gave the lowest average yield, the value of which (5.80% of D.M.) was not significantly different from that obtained in treatment 7 (5.68% of D.M.).

The results for corrected lignin are somewhat different here; the standard procedure (SM4) gave the highest result, whilst the modified main treatment (13) even when applied to material only extracted with ethanol-benzene gave a lower corrected lignin. The ash-free lignin from SM2 was higher than from the others because the absolute nitrogen content was about 3 times as high as in the ash-free lignin from the standard pretreatment (SM4). This indicates clearly the danger of relying on ash-free lignin without examining the quality of the lignin residue. The range between corrected lignin figures is much narrower than in the case of ash-free lignin (1.84 against 3.55). Comparison of SM3 with SM4 indicates that the 5% H_2SO_4 hydrolysis resulted in higher values for ash-free lignin and corrected lignin, due to the formation/

formation of residues insoluble in conc. HCl and 72% H_2SO_4. Comparing SM2 and SM3, the corrected lignin figures are closer than those for ash-free lignin, but the differences are appreciable in both cases. Since SM3 differs from SM2 only in the additional treatment with pepsin it is of interest that the recovery of nitrogen in the lignin residues is of the same order (about 36%). At least some of the nitrogen in the lignin from SM2 must have originated from protein material since it was extracted with pepsin in the case of SM3 and the similarity in nitrogen recovery suggests that the nitrogen in the lignin from SM3 may also be partly, if not largely, protein in nature.

A similar deduction was made by Thomas & Armstrong, (1949) using a different approach. But why is the corrected lignin from SM2 still higher than from SM3? It is unlikely that the pepsin-HCl treatment removes lignin from seeds-hay, because it was found that the absolute methoxyl content of SM2 was practically the same after pepsin-HCl digestion (Table A9). It is concluded that the lignin residue from SM2 contains non-nitrogenous interfering substances which had condensed with the lignin, or that the factor 6.25 used for protein correction was not applicable in all cases.

In the standard method of determination (SM4 and treatment 1) the recovery of nitrogen in the lignin/

lignin was twice as great as with treatment 13 due to the fact that the conc. HCl treatment removes some of the nitrogenous material.

The results are in favour of the modified main treatment applied to material extracted with ethanol-benzene and digested with pepsin-HCl; the lignin fraction obtained is perhaps the nearest to the true figure for lignin actually insoluble in 72% H_2SO_4. The lignin residue still contains some nitrogenous material and possibly other associates which condense with the lignin. The figures for corrected lignin seems to be more reliable than those for ash-free lignin.

Experiment (vi).

In this experiment (Table All) treatment 13 was again employed except that the digestion with 72% H_2SO_4 was carried out for varying times and only the starting material treated with both ethanol-benzene and acid pepsin (SM3) was used.

After digestion with strong acid for only 1 hour (treatment 14) filtration was possible although the cellulosic materials were still in a form reprecipitable by dilution. When the conc. HCl washings were diluted a humin precipitate was obtained similar in character to that obtained by diluting 72% H_2SO_4 filtrates, so it seemed that removal of this humin substance by the conc. HCl is the major factor facilitating the filtration/

filtration of the 72% H_2SO_4.

Three hours contact with 72% H_2SO_4 (Experiment (v) treatment 13) gave significantly lower results than the shorter period of contact in treatments 14 and 15 (Experiment (vi)), but contact for 5 hours (treatment 16) did not appear to lower the ash-free lignin any further. Three hours contact was considered to be adequate and after this time the hydrolysed cellulose is then no longer reprecipitable on dilution. Later it was preferred to digest with strong acid until a negative cellulose reaction was obtained with the acid mixture (2 - 3 hours).

Experiment (vii).

Duplicate samples (SM3) in Gooch crucibles were washed with conc. HCl then successively with water, alcohol and ether, then dried and moistened with 6 ml. 2:4 72% H_2SO_4 : conc. HCl to disintegrate cellulose, followed by contact with 60 ml. 72% H_2SO_4 for 2 hours, finally filtering and washing (treatment 17, Table A12). Treatment 18 was similar but included a final hydrolysis for 2 hours with boiling 3% H_2SO_4, similar to the final hydrolysis commonly used in methods of lignin determination. Treatment 17 was similar to 7 (Table A9) which produced the lowest ash-free lignin, but had the additional pretreatment with conc. HCl, and it was thought that this might prove

superior/

superior to treatment No. 13 (Table A 10). /(Table A12)
The results,/however, do not recommend it, as the ash-free lignin (6.22% of original DM) was greater than in treatment 13, (5.11%). Final hydrolysis with 3% H_2SO_4 (treatment 18) reduced the yield of ash-free lignin.

Experiment (viii).

In treatment 19, 5g. samples of hay were Soxhlet extracted 6 hours with ethanol benzene (syphoning every 10 minutes), then digested overnight with 200 ml. 0.1 N HCl containing 1% pepsin at 40°C then washed in a Gooch with water then with conc. HCl, followed by water, alcohol and ether, and finally dried. It was not necessary to dry the pretreated sample before washing with conc. HCl. The dry residue was digested with 150 ml. 72% H_2SO_4 until a negative cellulose reaction was obtained, then filtered and washed first with cold and finally with hot water. In treatment 20, the procedure of treatment 19 was followed by 2 hours hydrolysis with 3% H_2SO_4.

The results in Table A 12 show satisfactory reproducibility for treatment 19 and although the ash-free lignin (6.20 $\pm$ 0.05% of original DM) was significantly higher than obtained by treatment 13 (5.80 $\pm$ 0.04%) this may be due to inadequate removal

of/

of interfering associates when large samples are washed with conc. HCl. Final hydrolysis with 3% H_2SO_4 (treatment 20) gave a lower yield of ash-free lignin but this was probably due to the removal of some of the lignin during the hydrolysis.

Experiment (ix).

In treatment 22 the procedure was essentially the same as treatment 19 and was applied to starting material pretreated as in the slightly modified procedure of Armitage et al (1948) (SM6); treatment 19 was also applied to starting material pretreated similarly but omitting the 5% HCl prehydrolysis (SM5). Comparison was made with the whole procedure of Armitage et al (slightly modified). The results are given in Table A12 ~~and in Table above~~ are compared with the standard procedure, and with the samples only ethanol-benzene extracted (SM2), then subjected to treatment 13 (which is analogous to 19). Table A11 The ash-free lignin from the standard procedure was higher than from treatments 19, 21 and 22. The lower yield of ash-free lignin was given by treatment 19 with starting material treated with benzene and trypsin (SM5); the omission of 5% HCl hydrolysis was advantageous. Using the modified main treatment the difference between the ash-free lignin from SM5 and 6 (Table 12) is less than the differences between the

ash-free/

ash-free lignin from SM3 and SM4 (Table A11). This is probably due to the fact that 5% HCl hydrolysis is more drastic than 5% H_2SO_4 hydrolysis; in the former case greater removal of the lignin as well as of associates is likely. This is obvious from the data in Table A8: introducing 5% HCl hydrolysis prior to trypsin digestion decreased the plant material by 38.7% of original dry matter (74.03 - 35.34), whilst 5% H_2SO_4 removed 26.4% (64.8 - 38.49). Another factor accounting for the small difference between the ash-free lignins from SM5 and SM6, is the use of trypsin digestion which itself leads to higher yields of lignin than when pepsin digestion is employed, probably due to the fact that acid pepsin treatment removes about 10% more non-nitrogenous associates than alkaline trypsin.

The procedure of Armitage et al (modified) gives less ash-free and corrected lignin than that of Ellis et al (modified), perhaps due to the removal of more nitrogenous material in the Armitage method and the removal of some lignin itself in the more drastic 5% HCl prehydrolysis. The 0.25% Na_2CO_3 solution appeared to be too weak to remove detectable amounts of lignin from seeds-hay, and the absolute methoxyl content of the ethanol-benzene extracted material/

material was practically unchanged after further digestion with trypsin.

The results of this experiment favour the use of digestion with pepsin rather than trypsin in the pretreatment and confirm that dilute acid prehydrolysis is objectionable.

Experiment (x).

In this experiment an examination was made of the reproducibility of the proposed procedure which is based on the assumption that lignin is insoluble in strong acids and avoids dilute acid hydrolysis in the pretreatment and dilution of the strong acid in the main treatment. This procedure will be termed 'Proposed method for insoluble lignin' or 'Proposed method 1'. Separate samples equivalent to 2g. air-dried seeds-hay were used for all tests.

The previous experiments showed that the main treatment (19) was most practicable and gave lignin residues likely to be mostly true lignin, insoluble in 72% H_2SO_4. To avoid any loss of lignin it was preferred to wash the final residue with cold water and not with hot water or 3% H_2SO_4. The starting material was treated as indicated in Experiment viii; the ethanol-benzene treatment was that found to be most satisfactory in the previous studies of organic solvent extraction, (section 1, this chapter and

section/

section 3 , Chapter V). Experiments vii and viii had indicated a possibility that 2 hours final refluxing with 3% H_2SO_4 reduced the lignin yield so this was further examined. It was also considered that although the final hydrolysis might reduce the amount of lignin, this might not be objectionable for use in digestibility studies by the lignin ratio technique, so long as the fraction of lignin determined is totally recovered in the faeces. Insoluble lignin or 'Method 1' was used for all tests.

(a) Reproducibility of Proposed Method 1, without final hydrolysis.

The agreement between replicates (Table A13) was unsatisfactory, in the first and second tests [5.41 ± 0.072 (4), and 5.48 ± 0.136 (5) percent ash-free lignin in original sample], the maximum deviations being 3.5% and 6.9% of the means respectively, and more satisfactory than the maximum deviations of results recorded for the commonly used methods of lignin determination which vary from 7% (Armitage et al, 1948) to 16% or more (Ellis, 1949, McDougal & De Long, 1948_a). The coefficient of variation was 2.6% in the first test and 5.5% in the second. The results obtained in tests 1 and 2 were not significantly different ($P > 0.50$) and so were combined to give a more reliable value for the ash-free lignin.

(b)/

(b) Reproducibility of Proposed Method 1, with final hydrolysis.

The agreement between the 7 replicates of test No. 3 (Table A13) was again satisfactory and even better than for the 9 replicates without final hydrolysis. The coefficient of variation was 1.91 and the statistical range was narrower than when final hydrolysis was omitted.

(c) Effect of Final Hydrolysis.

The figures for ash-free or corrected lignin when final hydrolysis was employed were significantly different from those obtained without final hydrolysis. ($P < 0.01$). Two hours refluxing with 3% H_2SO_4 reduced the ash-free lignin by 9.4% and the corrected lignin by 9.7%. The nitrogen ~~content~~ of the ash-free lignin (%) was practically unaffected by the final hydrolysis, which may indicate that the lignin fraction as a whole was degraded in this treatment. Crude protein removed in the hydrolysate amounted only to 0.056% of the original DM or 0.9% of the ash-free lignin. The hydrolysates gave slightly positive reactions with phenol reagent but negative results were obtained in the ninhydrin test for α-amino acids so this may indicate the presence of phenolic groups from lignin rather than from aromatic amino/

amino acids.

(d) Effect of nitrogen correction on variability of replicates.

The nitrogen content was determined in the whole lignin residue of a separate determination, while other replicates were ashed to get the ash-free lignin. The same 'crude protein' correction was then made to each replicate so that although the mean corrected lignin was lower than the mean ash-free lignin (by the amount of the 'crude protein' correction,) the standard deviation, maximum deviation and standard error of the mean remained numerically the same, and the relative error of the maximum deviation (as percentage of the mean) and the coefficient of variation increased owing to the lower value of the mean corrected lignin. Nevertheless this increase in variability was not high.

(e) General Discussion.

The results of treatment 13 and 19 (Experiments v and viii) were within the range obtained in this experiment, but when treatment 19 was used with trypsin digestion instead of pepsin digestion, the ash-free lignin value was significantly higher ($P = 0.028$). This is in favour of using pepsin digestion as previously indicated. With pepsin digestion the lignin values (ash-free or corrected) obtained/

obtained without final hydrolysis were significantly lower than those obtained by the modified procedures of Ellis et al and Armitage et al, and by the proposed method when pepsin digestion is omitted, confirming previous results. The reproducibility with proposed method 1 is more satisfactory than that obtained with the method of Ellis et al (1948), in Section 1 (Table A5a) where 5 determinations on hay gave a maximum deviation of 11.5% and a coefficient of variation of 8.57%.

Although the reproducibility favours the proposed method, the lignin residue obtained still contains nitrogenous material to an extent similar to that of lignin obtained by the Ellis method (modified) and more than in lignin obtained by the modified method of Armitage et al. Although this proposed method is likely to be better than the current ones as there is no objectionable pretreatment, the residue obtained is unchanged and insoluble in 72% H_2SO_4, and the procedure is shorter, yet the residue may still contain associates which have condensed with the lignin. This is certain in the case of some nitrogenous materials as the nitrogen content has served to trace them (when pepsin-HCl digestion was omitted), but the nature of the whole nitrogenous fraction of the lignin residue is still uncertain, and the

nature/

nature of the non-nitrogenous (e.g. carbohydrates) associates is not easily determined.

Further study of the method with other plant materials, (particularly young plants) is necessary and the nature of the lignin residue needs further examination.

Ash content in lignin residue (Tables A7 - A13)

The ash content of the lignin residue fluctuated widely as shown in Table A10 and Table 21 p.235.

Table

With SM2, SM3 and SM4 the absolute ash contents varied within comparatively narrow limits, compared with the corresponding percentages of ash in the lignin residue; figures with the trypsin-Na_2CO_3 digestion and 5% HCl hydrolysis used in SM5 gave very much lower contents, indicating clearly that the ash content is affected by the pretreatment. Later it was found that even omitting the 5% HCl hydrolysis, but retaining the Na_2CO_3 digestion still produced an ash content of the same order, and it was concluded that the alkaline treatment with Na_2CO_3 is very effective in removing inorganic salts insoluble in acids. In one case, using SM6 (Armitage et al. procedure, modified), the ash content was practically nil.

It was concluded that the ash in the lignin residue/

TABLE (21) Ash content of acid Lignin from Seeds-Hay.

Analysis	STANDARD Procedure Ellis et al modified (S.M.4)	S.M.4 and modified main treatment	S.M.3 and modified main treatment	S.M.2 and modified main treatment	Armitage et al procedure modified (S.M.6)	S.M.6 and modified main treatment
Range of absolute ash (% of DM)	1.04 - 1.32	1.01 - 1.28	1.26 - 1.49	1.42	0.0 - 0.21	0.37
Range of ash (% in lignin residue)	12.4 - 13.7	13.3 - 16.8	12.8 - 22.7	12.8 - 15.4	0.0 - 3.5	5.1

residue is a contaminant, and is unlikely to be bound with the lignin, except perhaps to a minute extent. This view was later confirmed by preparing alkali and ester lignins which were practically ash-less (Chapters IV and VI).

Determinations at low temperature.

It was observed (section 1 (P 138)) that when the cold (-10°C) strong acid (72% H_2SO_4) was allowed to warm to room temperature nothing was precipitated which might have been dissolved at low temperature, or which might be formed at room temperature through reaction with the acid. Secondly, after incubating straw with 72% H_2SO_4 for 2 hours at - 10°C, followed by 1 hour at room temperature, the cellulose material was not precipitated by dilution and gave no cellulose reaction. Since the first observation showed that there was no fear of reprecipitating anything by warming, so long as the acid was undiluted, a quantitative test was carried out to examine the effect of low temperature reaction on lignin yield. A sample of seeds hay was pretreated as in the proposed method 1 up to the stage of washing with cons. HCl, then digested with 72% H_2SO_4 for three hours at - 5°C, followed by 1 hour at room temperature; after filtration the residue was redigested for 2 hours with fresh 72% H_2SO_4, then filtered and washed

with/

with boiling 3% H_2SO_4. Another sample similarly pretreated was digested with 72% H_2SO_4 for three hours at room temperature, then for one hour at 5°C, after which it was filtered, washed with 72% H_2SO_4, redigested for two hours at room temperature, filtered and finally washed with boiling 3% H_2SO_4. The yields of ash-free lignin from the two treatments were 6.06% and 6.68% of the original dry matter, showing that cooling did not lower the lignin yield as claimed by Hilpert and Littman (1935), using straw. The yield of ash-free lignin insoluble in 72% H_2SO_4 may not be affected by lowering the digestion temperature.

Determinations using pretreatment with acetoacetic ester.

It was found previously that plant material treated first with conc. HCl and then with ethyl aceto-acetate, no longer reacted with phloroglucinol, although after treatment with 72% H_2SO_4 it still gave a residue. This was re-examined quantitatively, using the principles of the proposed method 1, suitably adapted. Conc. HCl alone can dissolve substances from the pretreated plant material, so it was decided that complete extraction with conc. HCl was necessary before using the conc. HCl + aceto-acetic ester, in order to see whether any further extraction resulted from the use of the ester. Duplicate samples/

samples were pretreated, extracted with conc. HCl, then washed and dried, moistened with conc. HCl, and the ester added (about 20 ml. per gy.); after 24 hours contact at room temperature, the solvent was filtered off and the plant material washed with the ester, then successively with alcohol (to remove traces of the ester) water, alcohol and ether. After digestion with 72% H_2SO_4 for 3 hours and filtration without dilution, the residue was finally washed with water and boiling 3% H_2SO_4. The results are shown in Table 22 below.

Extraction with aceto-acetic ester reduced the yield of ash-free lignin from 5.45% of the original sample (vide experiment x) to 3.9% in one case and 3.0% in another, indicating that the aceto-acetic ester does not quantitatively remove lignin as claimed by Lemmel,(1935) and confirming Virasoro's (1942, 1943) findings with wood. Between 29% and 45% of the ash-free lignin was removed by the ester, but Virasoro,(1942) recorded the extraction of 77% of Kŏnig acid lignin from saw-dust.

After pretreatment and extraction with con. HCl a further 17.8 mg. (1.78% of the original sample) of substance were removed by ester treatment; if this is added to the final lignin yield it gives a value of 5.68%, which is within the range of the acid lignin values/

TABLE (22) Effect of Aceto-Acetic Ester Extraction.

SAMPLE	Plant Residue after pre-treatment & conc.HCl extraction.	Wt after Aceto-Acetic ester extn.	Extracted by the Ester		Ash-free Lignin	Ash-free Lignin (Control)
	g.	g.	g.	%	%	%
1	0.3618	0.3440	0.0178	1.78	3.90	5.45
2					3. 0	
Average					3.45	

values (5.10 - 5.76%) obtained by the control treatment (Table A13). It appears therefore that extraction with aceto-acetic ester is probably specific for lignin.

Summary and Conclusions.

From a study of various pretreatments and main treatments avoiding dilution of the strong acid, a proposed method was devised on the basis of insolubility of lignin in strong acids. This procedure is preferable to the current methods because it is shorter and avoids objectionable pretreatment and gives satisfactory reproducibility.

Dilute acid hydrolysis either in the pretreatment or in the final treatment may remove a lignin fraction, and in addition produces materials insoluble in the 72% H_2SO_4; in the case of the seeds-hay used this insoluble material produced exceeded the substances removed by the treatment.

There was evidence that at least a part of the nitrogenous material in lignin is of protein origin. Correction for nitrogenous material in the ash-free lignin appeared to give a more reliable figure, but the validity of the factor 6.25 is questioned, and the presence of non-nitrogenous contaminants is highly probable. The proposed method does not prevent the contamination of lignin by associates which can

condense/

condense with lignin. The need for further study of the quality of the lignin residues and of the application of the proposed method to young plants was indicated.

The whole of the ash present in the lignin residue was found to be a contaminant, so that it is unsound to record lignin constituents as percentages of the whole lignin fraction. After correcting for ash, the lignin constituents (e.g. nitrogen and methoxyl) expressed as percentages of the ash-free lignin are more reliable, although for comparative purposes it is also desirable to calculate the absolute amounts of N, MeO etc. by expressing them as percentages of the original plant material. Comparisons based on the percentage in the ash-free lignin may be unsound when contaminants are present.

Cooling the 72% H_2SO_4 during digestion did not appear to lower the lignin yield.

Aceto-acetic ester appeared to be a specific solvent for lignin, meriting further investigation.

CHAPTER IV

The Preparation of Alkali Lignin from Plants and a Study of its Properties in Relation to Acid Lignin.

In order to study the qualities of acid lignins and other residues (e.g. humin precipitates) obtained in the course of acid lignin determination a more or less 'pure' lignin was needed as a standard. Brauns 'native lignin' although prepared by mild conditions, represents only a small fraction of the total lignin, and from the study of organic solvent extractives, (Chapter V) it seems highly probable that Brauns extraction procedure for wood is unsuitable for feeding stuffs, particularly young plants. Bondi and Meyer[5] (1948) studies with alkali lignin from forage crops suggested that the quality of lignin was characteristic of the plant species from which it was obtained. As their method of extraction appeared to be much milder than that usually used for the extraction of alkali lignin from wood, the the yield obtained was high (about 5% of the dry matter); the preparation of alkali lignin was first adopted in an attempt to obtain a relatively pure 'reference lignin'.

As a result of experience with wood it has usually been claimed that alkali extraction is unsuitable for the quantitative determination of lignin, but it appeared that the alkali extraction of lignin from/

from plant material might be better than from wood. Figures recorded for the acid lignin in young plants are usually below 5% (Armitage et al, 1948, McDougal and De Long, 1948a,b,c) and for hays about 7% (Armitage et al, 1948: Chapter III sections 1 and 4). Bondi and Meyer's yield of alkali lignin was not much less and considering the possibility of acid lignin remaining in the plant material after alkali extraction, it seemed possible that if both the alkali lignin and the residual acid lignin were determined the summation might give a quantitative measure of the total lignin. Extraction of plant material with alkali also removes proteins and hemicelluloses very effectively so it was considered possible that the alkali extraction might serve as a good pre-treatment leading to the production of an acid lignin of high quality, possibly suitable for a reference lignin. The comparison of such acid lignin with that prepared by alkali from the same plant, and with the lignin obtained by current methods seemed likely to yield useful information on the nature of lignin.

The determination of the methoxyl content of the lignin residue in addition to the N content was considered essential for satisfactory comparisons as it may be a better index of lignin purity. Also in/

in the examination of different lignin preparations f from the same sample, the absolute methoxyl contents of the lignin residues serve to indicate the relative amounts of pure lignin present in them; so giving a sound basis for comparative study, particularly if the pretreatments were effective in removing 'labile methoxyl or ester methoxyl' from the plant material. Comparisions on the basis of methoxyl content are, however limited to individual samples since the literature indicates (although without clear-cut evidence) that the percentage of methoxyl in plant lignin increases with the age of the plant and varies according to the species. (Chapter I p. 27)

4.1. Plant material and procedures.

Seeds hay was used for the majority of the experiments but an air-dried sample of young red clover (3 weeks growth, cut in July 1949 and having a high N content) was also used, the lower part of the stems being discarded in order to give a more homogeneous sample of the succulent tissues and leaves. For confirmatory experiments and special purposes samples of oat straw, mature lucerne, immature lucerne (before flowering), immature pasture herbage (containing 37% legumes) and sheep's faeces were used. The freshly cut immature lucerne was minced and washed several times with water on a cheese/

cheese cloth in a Buchner funnel then dehydrated with alcohol (which also removes pigments, particularly chlorophyll), then finally with ether. The other samples were oven dried (100-105°C) and finely milled. Ethanol-benzene extraction was then carried out. The nitrogen in the dry matter of the samples used is given in Table A14.

The procedure for alkali lignin extraction was based on that of Bondi and Meyer (1948), modified as in the appendix p. 8 . Two preliminary trials were made with seeds hay. In the first (Treatment 1, Table A14) the Bondi and Meyer procedure was modified in an attempt to make the conditions milder. To avoid possible changes 0.5N NaOH plant extracts (at 85°C) and water washings were not evaporated and the final evaporation of the slightly acid alcohol solution containing soluble lignin was carried out under reduced pressure to lower the temperature and avoid possible reaction between the alcohol and the lignin. After most of the alcohol was removed small amounts of water were added and redistillation under reduced pressure continued to remove the last traces of alcohol and ensure more quantitative separation. It was found that if after the first treatment of the plant material with 0.5N NaOH for 8 hours at 85°C, the filtered residue was washed with 0.5N NaOH rather than/

than water, it was more efficiently washed so that in the second and third treatments only negligible precipitates of lignin were obtained and only a single extraction with 0.5N NaOH became necessary.

In the second preliminary trial (treatment 2, Table A 14) when the residue precipitated by the addition of alcohol to the alkali extract (Bondi and Meyer's residue 1, containing salts and hemicelluloses and some lignin) was treated for a further yield of lignin, the amount obtained was less than 1.5% of the amount obtained from the first alcohol solution. It was also observed that the rapid addition of 5N H_2SO_4 (with stirring) to acidify the alkaline lignin solution gave a colloidal precipitate of lignin which passed through Whatman No.50 filter peper. This filtrate was made alkaline to dissolve the dispersed lignin, then acidified gradually and left overnight when a humin residue came down. Heating (on a water bath) coagulated and darkened this residue which settled down leaving a clear supernatant solution; the filtrate remained clear when left for 24 hours. When reprecipitating alkali lignin, therefore, slow mixing of the precipitating agent, and indirect heating with a water bath, warmed gradually from cold, was necessary to avoid the formation of a colloidal precipitate. This procedure caused the lignin residue to coagulate as large firm flakes easily filtered/

filtered, leaving a clear supernatant liquid.

Filtration of the final lignin solution in warm 1.25N NaOH (after removal of alcohol) was necessary because a small fraction of the residue resisted solution in the alkali.

No residue was produced when either protein, (casein) or pectin was incubated for 8 hours at 85°C with 0.5N NaOH, then passed through the alkali lignin extraction procedure and alcohol added. The possibility of condensation occurring with the lignin in alkali solution cannot, however, be excluded.

The extracted pulp was washed with water then successively with 1% HCl, water, alcohol and ether. In some cases it was then ground in a small hand mill which enabled a quantitative collection of the residue.

After alkali extraction the residue (pulp) was treated with 72% H_2SO_4 until the reaction with I_2-H_2SO_4 was negative, then after dilution 5-6 times and filtration through Whatman No.50 paper the residue was transferred to a sintered glass crucible and washed with boiling 3% H_2SO_4. This produced acid lignin in a manner similar to that used in the current main treatments but without the final hydrolysis. Besides removing lignin the alkali treatment was very efficient in removing associates from the plant material. It was also found that

when/

when the strong acid filtrate obtained from treatment of the pulp was diluted, it gave only negligible amounts of humin residue so that in this case it was not necessary to avoid dilution before filtration.

4.2 Experiments with seeds-hay consisting largely of Ryegrass (Sample 2).

Experiment (1)

(a) Yield and composition of lignin

In this experiment treatments 1, 2 and 3 shown in Table A 14 were employed. In treatments 1and 2 the plant material was pretreated as in the proposed method 1, but in treatment 3 only petroleum ether extraction (similar to the ether extraction used by Bondi and Meyer) was employed in order to see the influence of pretreatment on the quality of the alkali lignin obtained.

In treatment (1) where the extracted pulp was washed with water the yields of lignin from the first and second extractions were 2.5% and 0.7% of the original dry matter, respectively, but in treatment (2) where the pulp was washed with 0.5N NaOH, the yield of lignin from the second extraction was negligible, justifying the modification of the procedure. The total yield was higher in Treatment (2) because the mode of precipitating and coagulating the lignin was better than in the first treatment and/

and the separation was more quantitative.

The qualities of the alkali lignins from treatments 1 and 2 were quite similar although apparently better in the first treatments, the nitrogen content being less and the methoxyl percentage higher. When ethanol-benzene extraction and pepsin digestion were employed (treatments 1 and 2) the lignin was of better quality than when only petroleum ether extraction was used. In the latter case the percentage of nitrogen in the ash-free lignin was 38-59% higher while the methoxyl percentage in the corrected lignin was 13 - 17% lower.

The methoxyl content of the alkali lignin is considerably higher than that obtained by Bondi and Meyer (1948) for alkali lignin from gramines (10%) and approaches the methoxyl percentage reported for Spruce native lignin, and alkali lignin from straw. The nitrogen percentage is above the lower limits reported by Bondi and Meyer (1948) for cereal lignins.

(b) Properties

(i) The alkali lignin from seeds hay has a characteristic manner of precipitation from alkaline solution on acidification and exhibits colloidal properties changed by heating as referred to above. When first precipitated from the alkaline solution the/

the lignin is lighter in colour than after coagulation into firm flakes by heat; only the former can be dispersed in water. The colour and coagulation properties were found to be important characteristics, greatly affected by contamination: the more nitrogenous material there is in the lignin, the darker is the colour, and the less firm the coagulum. This is also shown by alkali lignins from young and leguminous plants.

(2) The solution in alkali is dark, giving a deep colour with phenol reagent.

(3) It gives the characterstic reaction with chlorine and sodium sulphite.

(4) The lignin from treatments 1 and 2 did not reduce Fehling's solution or yield furfural in the pentose test, but with the lignin from treatment (3) reduction of Fehling's solution did occur. From the methoxyl content and the absence of furfural-producing or Fehling reducing substances, it was concluded that the alkali lignin obtained in treatments 1 and 2 might be fairly pure.

(5) The Lassaigne test indicated the presence of nitrogen but sulphur and halogens were absent.

(6) The lignin is insoluble in ether, benzene and acetone and almost insoluble in conc. HCl. Bondi and Meyer (1946) obtained alkali lignins which were insoluble in acetone. The lignins here were partially soluble in glacial acetic/

acetic acid and 72% H_2SO_4 and soluble in α-naphthol (decreasing its melting point) alcohol and melted trichloracetic acid (reprecipitated from the latter by dilution with water); they dissolved in cold ethyl aceto-acetate when moistened with conc. HCl, but were insoluble in the cold ester alone although a small portion appeared to dissolve in the boiling ester.

(7) The lignin burns with a smoky flame similar to that of aromatic compounds, and leaves no residue on ignition.

(8) It dissolved completely in conc. H_2SO_4 and dilution did not cause reprecipitation. When the diluted solution was made alkaline, it gave a positive reaction with phenol reagent but the colour appeared to be less than that obtained with the original alkali lignin. With a greater concentration of lignin, in conc. H_2SO_4 for 24 hours it still dissolved, but on dilution a humin residue was obtained leaving a turbid supernatant solution. The humin residue mostly passed through a sintered glass crucible and when heated in 3% H_2SO_4 did not coagulate but dispersed or dissolved, no precipitation occurring on standing. It was thought that sulphonation may have occurred, but the addition of $AgNO_3$ to the solution did not bring about precipitation, suggesting the absence of sulphonic acids (Siggia, 1949, p.99).

The/

The common assumption that lignin is insoluble in all concentrations of sulphuric acid seems to be untenable. Concentrated H_2SO_4 may possibly produce depolymerization.

(9) On treatment with 72% H_2SO_4 the alkali lignin partially dissolved but the major part remained insoluble and darkened in colour. On dilution the soluble part separated out as a humin precipitate which resisted boiling with dilute acid; prolonged boiling produced granulation in a way similar to the humin precipitate obtained on dilution of the 72% H_2SO_4 filtrate in the acid lignin determination. Only very slight dilution of the 72% H_2SO_4 containing the soluble part of the alkali lignin (0.1 - 0.3 ml water per ml acid) led to the separation of the alkali lignin in humin form, and this precipitate behaved exactly like the humin precipitate obtained from 72% H_2SO_4 filtrates in the earlier work with acid lignin (Chapter III P.164). The quality of the humin precipitate from the soluble part of the alkali lignin was very similar to that of the insoluble part and to the original lignin, as shown in the figures below, extracted from Table A 15.

This behaviour with 72% H_2SO_4 showed a close relationship between alkali and acid lignins and supported the view put forward previously that the humin precipitates obtained in acid lignin determination are/

Material	Nitrogen % of lignin	Methoxyl % of lignin.	Methoxyl % of corrected lignin
Original alkali lignin	1.49	14.4	15.9
Fraction soluble in 72% H_2SO_4	1.35	13.91	15.2
Fraction insoluble in 72% H_2SO_4	1.38	14.1	15.4

are related to lignin itself. It seems highly probable that during acid lignin determination, part of the lignin remains in solution or highly dispersed in the 72% H_2SO_4.

The part of the alkali lignin insoluble in 72% H_2SO_4, was treated with NaOH but only dissolved slowly and incompletely, even when heated with strong NaOH solution. Moreover when washed with H_2O extensively, then dried, this alkali lignin insoluble in 72% H_2SO_4 acquired solubility properties similar to the dry acid lignin prepared by the modified method of Ellis et al; it was insoluble in the common organic solvents, almost insoluble in cold alkali and only partially soluble in boiling NaOH, melted trichloracetic acid or ethyl aceto-acetate plus conc. HCl. The changes in the physical properties of lignin brought about by the addition of 72% H_2SO_4 were thus clearly demonstrated and changes in solubility may be important in governing chemical reactivity. It was observed previously

(p/

(p.206 text) that alkali lignin treated with 72% H_2SO_4 was no longer chlorinated in the same manner as the untreated alkali-lignin; the latter reacted quickly forming soluble chlorolignins, whilst the former remained largely insoluble, even after the addition of the alkaline Na_2SO_3 solution. These findings throw doubts on the results of Sowden and De Long (1950 a and b) based on the comparison of acid lignins prepared from wood (as reference) with those from young plants, after chlorination and sulphonation.

Experiment ii Behaviour of Alkali Lignin under conditions of acid Lignin determination (Table A15)

Treatment 1. About 500 mg of alkali lignin from seeds hay were treated with 20 ml 72% H_2SO_4 for 3 hours at 20°C with occasional stirring. Filtration through a sintered glass crucible (porosity 3) was possible without dilution because the insoluble particles were coarse and the insoluble material was then washed successively with 72% H_2SO_4, water, and boiling 3% H_2SO_4. The soluble lignin in the filtrate was precipitated by dilution about 5 times but the precipitate was not retained by a sintered glass crucible (porosity 3). When diluted to 3% H_2SO_4 and heated to boiling point the precipitate coagulated and the major part was then retained by the same crucible, although the filtrate was turbid. Refiltering/

Refiltering through a Gooch crucible and washing with water gave a clear yellowish filtrate; the fraction retained by the Gooch was determined by ignition. The yellowish filtrate when made alkaline gave a positive reaction with phenol reagent showing that lignin was present.

The fraction insoluble in 72% H_2SO_4 amounted to 69.2% of the original alkali lignin (Table A15) whilst the portion recovered from the fraction soluble in 72% H_2SO_4 was 14.5% indicating a loss of about 16% of the staring material. Thus in an ordinary acid lignin determination, a loss of some of the humin lignin fraction is to be expected. It should be noted that in the ordinary acid lignin determination filtration is more difficult if the diluted 72% H_2SO_4 extract is not boiled (e.g. in the first filtration made in the 'final treatment' of Ellis et al procedure).

The effect of 72% H_2SO_4 on the physical and analytical qualities of alkali lignin have been mentioned in Expt. 1 above. It should be noted here that the total recovery of alkali lignin in treatment 1 (83.7%) was very similar to the total recovery of methoxyl (82.0%) indicating that the methoxyl content of the unrecovered fraction was similar to that of the recovered lignin; it is probable that treatment with 72% H_2SO_4 separates alkali lignin into two fractions of/

of approximately the same methoxyl percentage, rather than produces demethoxylation. In the case of nitrogen, however, the recovery was (74.5%) lower than that of the lignin (83.7%) indicating that although most of the nitrogenous material in lignin resisted 72% H_2SO_4, some hydrolysis of the nitrogenous material may have occurred.

Treatment 2 Triplicate samples (116-200mg) of alkali lignin were each treated with 72% H_2SO_4 (20ml at room temperature) in the presence of 1 g ashless filter paper (finely cut) until the cellulose reaction was negative, and then diluted about 5 times and treated differently as follows:- (2.1) filtered through a Gooch crucible, then washed with boiling 3% H_2SO_4. A very slight humin precipitate was washed out in the 3% H_2SO_4 filtrate and the recovery of the alkali lignin was 97.5%. (Table A 15)

(2.2) filtered through a Gooch crucible but washed with water. The fraction retained amounted to 76.85% of the original lignin, an appreciable amount of the humin lignin being washed out with the water; on boiling this humin fraction with 3% H_2SO_4 and refiltering through a Gooch, then washing with water, a clear yellowish filtrate was obtained and the residue retained in the Gooch amounted to 15.35% of the original alkali lignin. The total recovery (92.16%) was lower than in treatment 2.1 and the boiling/

boiling appeared to dissolve some of the lignin. Whereas the acid medium in treatment 2.1 appeared to keep the humin lignin undispersed, water alone either dissolved or dispersed part of it.

(2.3) filtered through a Gooch, washed with 3% H_2SO_4, then finally refluxed 2 hrs in 3% H_2SO_4, refiltered through a Gooch crucible and washed with water. Thefiltrate remained clear but yellowish. The recovery of lignin was only 85.13% in this treatment which was similar to the final treatment in the modified procedure of Ellis et al. The boiling in dilute 3% H_2SO_4 dissolved some lignin and lowered the recovery. Russel (1948) did a Klason lignin determination on a purified alkali lignin from corn cobs and obtained a yield of 88.3%.

Treatment (3.1) A sample (about 425 mg) of alkali lignin was treated with 72% H_2SO_4 in the presence of pure cellulose as before but for a longer period (6 hrs.), then diluted, filtered through a sintered glass crucible (porosity 3) and washed with boiling 3% H_2SO_4 and water. A small humin residue came down in the filtrate but the lignin retained by the crucible amounted to 95.3%. In treatment 1, the humin fraction passed through a sintered glass crucible and the higher retention in treatment 3.1 was probably due to part of the humin lignin adhering to or being adsorbed by the lignin fraction insoluble in/

in 72% H_2SO_4. The filtrate was refiltered through a Gooch and washed with boiling 3% H_2SO_4, increasing the total recovery of lignin to 96.47%, similar to treatment 2.1. Treatment 3.1 had some effect on the quality of the lignin, lowering the percentage of methoxyl from 14.4 to 13.77.

Treatment (3.2) The dried residue from treatment 3.1, was further refluxed for 2 hours with 3% H_2SO_4, filtered through a sintered glass crucible and washed with water. The recovery (99.54%) was practically complete. Comparison with treatment 2.3 shows that the freshly prepared wet residue from 72% H_2SO_4 is more susceptible to hydrolysis than the dried residue, probably because of the physical changes produced by drying.

Treatment (3.3) The residue from treatment 3.2 was further subjected to vigorous hydrolysis with 5.0N H_2SO_4 under a reflux for 24 hours. The recovery was 86.2% but the quality of the lignin was changed. The nitrogenous material was reduced to about one third, and methoxyl to two thirds. If allowance is made for these changes in composition and the recovery of the methoxyl-free fraction of the corrected lignin calculated as shown below, it is seen that this recovery is very high (95.7%)

The lignin molecule was not broken down as a whole; a resistant 'skeleton' remained while demethoxylation/

Fraction	% Crude Protein in Lignin	% Corrected Lignin	% Methoxyl in Lignin	% Methoxyl-free Corrected Lignin
(A) Starting Material (170.1mg)	9.62	90.38	13.77	76.61
(B) Recovered Material (146.7mg) Per cent	8.75	96.25	11.31	84.94
Per cent of (A)	8.25	83.01	9.75	73.26
Percentage Recovery	33.6	91.8	70.9	95.7

demethoxylation and removal of nitrogen took place. It is unlikely that the nitrogen is firmly bound in the aromatic nucleus of lignin as claimed by Bondi & Meyer (1948) who used similar hydrolytic treatments and claim that the nitrogen content of the lignin was not diminished. The results here are in accordance with those of Thomas & Armstrong (1949) who were able to remove 50% of the nitrogenous material from alkali lignin (prepared by the procedure of Bondi & Meyer) by 5 hrs. hydrolysis with 2.5 N-hydrochloric acid at 15 lb. pressure.

The ninhydrin test for α-amino acids gave a positive reaction with the 5 N-H_2SO_4 hydrolysates from alkali lignin. (The theoretical amount of $BaCl_2$ was added to remove the sulphate ion, then the

filtrate/

filtrate was evaporated under reduced pressure with the addition of portions of water to facilitate removal of hydrochloric acid; the test with ninhydrin in buffer solution was applied to the final few drops of concentrated filtrate.) Similar results were obtained later (Chapter V, p. 314) with acid lignin prepared from young clover and containing a much higher nitrogen content, but in that case 87.3% of the nitrogenous material was removed by hydrolysis.

Thomas & Armstrong identified several amino acids in their acid hydrolysate and it is safe to conclude that at least part of the nitrogenous material in lignin is of protein origin as indicated by the earlier results (p. 223). It is very unlikely that the nitrogen is bound in the aromatic nucleus, but the possibility cannot be ignored that there may be some kind of combination between nitrogenous material and lignin accounting for the resistance to 72% H_2SO_4 and mild hydrolysis.

Experiment iii Acid Lignin from Seeds-Hay Alkali Pulp.

In treatments 2 and 3 of Tables A14 and A16 the yield of acid lignin (ash-free or corrected), obtained from the pulp remaining after removal of alkali lignin (Table A16) was equal to about one-third of the yield of alkali lignin (Table A14) but the/

the yield of methoxyl was only about one-fourth of that in the alkali lignin. The nitrogen content of the acid lignin from the pulp was much lower than in the alkali lignin; it was also lower than in the acid lignin obtained by the modified procedure of Ellis et al, which is not surprising in view of the almost negligible nitrogen content of the pulp, the pretreatment and alkali extraction removing 97.4 - 98.7% of the absolute nitrogen content of the original plant material. Of the remaining nitrogen (1.3 - 2.6%) 30 - 60% was recovered in the lignin itself, the lowest nitrogen in the acid lignin (0.3%) being similar to the nitrogen in acid lignins prepared from soft woods. These results indicate that the whole of the nitrogenous material in the lignin fraction is probably a contaminant. From the same plant material acid lignin prepared by proposed method 1 after extraction only with ethanol-benzene, contained 4.46% nitrogen and about 20 different acid lignin preparations were made from that hay (Chapter V) containing from 0.3% to 5.64% N according to the pretreatment, the nitrogen content of the material treated with 72% H_2SO_4 and the contamination with non-nitrogenous material. Contamination with nitrogen is thus similar to contamination with mineral matter.

The methoxyl percentage in the acid lignin from the/

the pulp (Table A16) was significantly lower than in the alkali lignin (Table A14), and similar to that in acid lignin prepared by the modified method of Ellis et al (Table A18). This shows that despite the very efficient pretreatment provided by alkali extraction, the acid lignin from the pulp still contains non-nitrogenous contaminants which lower the methoxyl percentage. It is unlikely that demethoxylation takes place during alkali treatment since the alkali lignin, which, being dissolved by the alkali was more susceptible to chemical change, contained a higher methoxyl content. The 72% H_2SO_4 is also unlikely to produce demethoxylation as indicated by its effect on alkali lignin (p.256 Text). Although Phillips et al (1939) found that the percentage of methoxyl in the acid lignin (prepared by the A.O.A.C. method) from one part of the plant(e.g. leaf) differed from that in the lignin from another part (e.g. stem), supporting the view that several lignins may be present in a single plant, it seems unlikely that alkali would remove a type of lignin rich in methoxyl and leave in the pulp a type poor in methoxyl. The pulp acid lignin, representing the more resistant lignin, might be expected to be derived mostly from the stemmy parts of the plant, but according to the results of Phillips et al (1939) it should therefore contain a higher methoxyl content. The results obtained here are therefore not in harmony/

harmony with the findings of Phillips et al (1939). Both the acid lignin obtained from seeds-hay by the method of Ellis et al and the pulp acid lignin were contaminated with non-nitrogenous materials, and it seems that the alkali lignin from seeds-hay is purer and a better reference substance. It should be noted that the alkali lignin is still contaminated with nitrogenous materials to a greater extent than the acid lignin from the pulp, and there seems to be no direct relation between the nitrogen and methoxyl contents of lignin; the nitrogen content alone is an inadequate index of purity and figures for corrected lignin (i.e. ash-free lignin - N x 6.25) are therefore of questionable validity.

In Table A17 the summation of the values obtained for the alkali lignin (Table A14) and for the acid lignin from the alkali pulp (Table A16) are compared with the values for acid lignin obtained by the modified procedures of Ellis et al (1946) and Armitage et al (1948). The summation figures are of the same order as those for the Armitage method, somewhat lower than for the Ellis method, and higher than for the proposed method 1 without final hydrolysis (Table A13). The summation figures for absolute methoxyl content were higher than found in the Armitage method but similar to the figures for the Ellis procedure. This may indicate that the summation gives/

gives a value within the limits of the current methods of determining acid lignin and an even more absolute measure of lignin content (judged by absolute methoxyl) may be obtained; however the two stage determination of lignin is not suitable for routine analysis, and mechanical losses of lignin are almost certain to occur.

4.5. Experiments with Young Clover

i Alkali Lignin

At first (Table A14, treatment 4) lignin was extracted from clover treated only with ethanol-benzene in order to compare the results with those of Bondi & Meyer (1948). The lignin obtained had a nitrogen content about double that recorded by Bondi & Meyer (2.92-3.36) for young legumes whilst the methoxyl content, even after correction for nitrogen, was well below the 5% level which Bondi & Meyer suggested as characteristic of legumes. The yield of alkali lignin was low, it was dark in colour, and did not give a firm clean coagulum like the alkali lignin from seeds-hay.

The experiment was repeated (Table A14, treatment 5) with pepsin digestion after the ethanol-benzene extraction and using a milder alkali extraction of the pretreated residue (with warm 0.5 N NaOH); the alkali extract was acidified and treated as in the modified Bondi & Meyer procedure. The yield of alkali lignin was much lower, but the nitrogen content was reduced and/

and the methoxyl content increased. The nitrogen percentage was still relatively high (6.34%) however, and even alkali lignin prepared from a 0.25% Na_2CO_3-trypsin hydrolysate of ethanol-benzene extracted clover (Table A14, treatment 6), contained high nitrogen and low methoxyl percentages. These results are not in accordance with those of Bondi & Meyer (1948) and indicate that alkali lignin is not of constant quality. Enzymatic digestion combined with milder conditions of alkali extraction improved the lignin quality. The Clover plants used by Bondi & Meyer were of lower nitrogen content and more advanced in growth (1.5 - 2.5 months in a Mediterranian climate). Recorded figures for acid lignin from young plants show that in some cases the nitrogen content is higher than obtained in treatment 5 (Table A14) whilst the methoxyl percentage is of the same order. The modified Ellis et al procedure gave with the same clover an acid lignin containing about half the nitrogen percentage, and double the methoxyl percentage (Table A18) and so was of better quality than the alkali lignin in contrast with the results obtained with seeds-hay (also shown in Table A.8).

ii Acid Lignin from the Pulp.

This was of better quality than either the alkali lignin or the acid lignin obtained from the whole/

whole plant by the method of Ellis et al (Table A18). The nitrogen percentage (1.1) was much lower and the methoxyl percentage was over 10%, and comparable with the amount in the pulp acid lignin from the seeds-hay. Although the two pulp acid lignins appeared to be of similar quality the nitrogen content was higher in the case of the clover. On account of its relative purity the acid lignin obtained from the pulp appears to be a better reference lignin than either the alkali lignin or the acid lignin prepared by current methods, but it may nevertheless be contaminated with non-nitrogenous materials and the 'pure lignin' from young clover may contain more than 11.4% methoxyl. The results obtained at this stage threw doubt on the prevailing view that the methoxyl content of lignin is low in young plants and increases gradually with age and further evidence on this point was obtained later (Chapter VI).

The summation of alkali lignin and pulp acid lignin from clover is compared with the acid lignins obtained by the modified methods of Ellis et al and Armitage et al in Table A 17. The total figures for ash-free and corrected lignin are somewhat lower than those for the Ellis method, but higher then those for the Armitage method, and the figures for total methoxyl show similar differences.

Whereas/

Whereas for seeds-hay the yield of alkali lignin was more than three times the yield of pulp acid lignin, in the case of clover the yield of ash-free pulp acid lignin was equal to the alkali lignin, whilst the yield of corrected acid lignin from the pulp was 1.8 times as great as the corrected alkali lignin. At the same time the methoxyl content of the pulp acid lignin from clover was many times greater than that of the alkali lignin (Table A17) and the absolute (% of original D.M.) methoxyl content of the pulp acid lignin was almost as great as that of the acid lignin prepared by the Armitage method. It seems that the lignin in young clover is more resistant to alkali than that in seeds-hay consisting largely of Italian ryegrass.

The recovery of the pulp methoxyl in the acid lignin extracted from the pulp was about 66% in the case of seeds-hay and 77% for clover (Table A16), although extraction with alkali had removed most of the hemicelluloses. In the case of wood the recovery of methoxyl in the acid lignin is much higher, (Dorée (1947, p.367) gives 91-97% of the original methoxyl in the wood), and in the treatment with 72% H_2SO_4 of alkali lignin from seeds-hay (Table A15, treatment 2), (Section 2 p.256), 97.51% of the lignin was recovered and the percentage of methoxyl in the lignin/

lignin was possibly not significantly affected by treatment with 72% H_2SO_4 (note treatment 1 Table A15). Dorée (1947) page 367) considered that with wood the losses of lignin methoxyl due to the effect of H_2SO_4 were not so high as they appeared (3-9%) because some of the wood methoxyl was present in the hemicelluloses. (See also Norman, 1937, p. 41). This is in accordance with the experimental results from the treatment of seeds hay alkali lignin with 72% H_2SO_4. The lignin in the pulp is likely to be more resistant than the fraction removed by alkali, and is probably more highly polymerized so that the recovery of the acid lignin from the pulp should be greater than the recovery of alkali lignin, and in fact should be practically 100% provided there are no losses in filtration. Under these conditions therefore any methoxyl lost by treatment of the pulp with 72% H_2SO_4 must belong almost entirely to non-lignin material (the holocellulose fraction of the pulp). In the preparation of the acid lignin from the pulp, a sintered glass crucible was used for the final filtration, and washing was with boiling 3% H_2SO_4, so that some loss of the humin fraction of the lignin may have occurred although in later experiments (Chapter VII) when precautions were taken to avoid any loss of lignin the recovery of methoxyl was nevertheless only 80.9%. Although the absolute amount/

amount of the humin fraction is small (0.21 to 0.25% of the original seeds-hay) it forms 10 to 13% of the pulp acid lignin obtained (See footnotes to Table A16). The holocellulose methoxyl appears to be firmly bound as it resists the alkali extraction. Phillips et al (1939) also found that the methoxyl recovered in the acid lignin was lower than the methoxyl in pretreated oat plants, and attributed the loss to carbohydrates containing firmly bound methoxyl.

4.4 Experiments with other plant materials

(i) Straw The alkali lignin obtained from oat straw was of the best quality, containing a higher percentage of methoxyl/than the Willstatter lignin (Table A14) prepared by Phillips et al (1939) from mature oats, and probably higher than any figure recorded for alkali lignin from cereal straws. It seems that alkali extraction in the absence of nitrogenous materials can produce lignin of high quality. It was lighter in colour than the alkali lignins from seeds-hay or faeces from sheep consuming timothy hay and on acidification and heating, the solution in alkali gave a firm coagulum and left a transparent supernatant solution.

(ii) Lucerne With both mature and immature lucerne the yields of alkali lignin were low and the quality poor, particularly in the case of the immature/

immature material which gave an ash-free lignin containing 9.76% N (61% crude protein) and a corrected lignin containing only 6.59% methoxyl (Table A14). The corresponding figures for the lignin from mature lucerne were 6.97% N and 7.51% methoxyl. The alkali lignin obtained in each case was similar to that from clover, being dark in colour, and not giving a firm coagulum. The presence of nitrogenous material soluble in alkali appears to be responsible for much of the contamination of the alkali lignin obtained from plants, and the higher the temperature used in alkali treatment the greater the condensation of nitrogenous constituents and the greater the contamination. Although the young clover sample contained more nitrogen than the immature lucerne the alkali lignin obtained from it contained a lower nitrogen percentage than the alkali lignin from the immature lucerne because in the case of lucerne boiling with 0.5N NaOH was employed.

(iii) Faeces The alkali lignin from faeces of sheep fed on Timothy hay contained a high methoxyl content similar to that of the alkali lignin from sheds hay (ryegrass) but the nitrogen content was somewhat higher (Table A18). The ash-free acid lignin from the alkali pulp contained 10% methoxyl compared with 10.22% for the ash-free acid/lignin obtained by the modified procedure of Ellis et al. The faeces from sheep/

sheep grazing immature pasture (containing 63% grasses, 37% clover) had a nitrogen content intermediate between those of legumes on the one hand and cereal straws and hays on the other. Successive extractions of alkali lignin were made, as indicated in treatment 11 of Table A14. In treatment 11A the ethanol-benzene extracted faeces were extracted with 0.5N NaOH at 85°C and washed with water. The alkali lignin obtained contained less nitrogen and more methoxyl than that obtained from mature lucerne but the quality was poorer than that of the alkali lignins from seeds-hay or from the faeces of sheep fed on timothy-hay. From a second extraction under the same conditions (treatment 11B), a smaller yield of alkali lignin was obtained with a lower nitrogen percentage but similar methoxyl percentage. The lower nitrogen content supports the view that a combination or reaction of some sort occurs between soluble nitrogenous materials and lignin; as the first extraction removed the greater part of the nitrogenous material, in the second extraction there would be relatively little available for condensation with lignin.

The acid lignin from the pulp (Table A 18) contained a lower nitrogen percentage and a slightly higher methoxyl percentage than either the alkali lignins obtained from treatments 11A and 11B (Table A14)/

Table A14) or the acid lignin prepared by the Ellis method (Table A18) due no doubt to the efficiency of the alkali treatment in removing associates. The pulp remaining after the two alkali extractions was oven dried; part of it was used to prepare acid lignin (Table A16) whilst another part (treatment11C, Table A14) was first extracted with acetoacetic ester to give a yield of ester lignin, then the ester pulp further extracted with boiling 1.25N NaOH for 40 minutes to give another preparation of alkali lignin.

It will be seen from Table A18 that the quality of the pulp acid lignin (as indicated by methoxyl content) is fairly uniform, whether obtained from seeds hay, young clover, or different types of faeces but this is not the case with the alkali lignin. The alkali lignin from material of high nitrogen content (e.g young clover) was of very poor quality due to the presence of contaminants soluble in the alkali, whereas after the removal of the greater part of these contaminants by alkali treatment a pulp acid lignin is obtained with much higher methoxyl content and comparable with that from other materials.

The ester lignin from the pulp was found to be of better quality than either the alkali lignin or the acid lignin prepared from the same pulp as shown by the figures below (p274). The nitrogen percentage was only/

(see over for table)
only about a fourth of that in the alkali lignin and about two fifths of that in the pulp acid lignin whilst the methoxyl percentage was more than 50% greater than in the alkali and pulp acid lignins. Thus the latter lignins must be more contaminated with associates so that neither alkali lignin nor pulp acid lignin may be regarded as reliable reference lignins. The high methoxyl percentage found in ester lignin is contrary to the common view that the methoxyl content of the lignin in immature plants is low.

The high methoxyl content of the ester lignin suggests that the acetoacetic ester is a more selective solvent for lignin, so giving a less contaminated product. If the ester was producing partition of the lignin with preferential solution of a fraction high in methoxyl the residual lignin would be expected to be very low in methoxyl. This was not the case, however, the alkali lignin (ash-free) obtained from the pulp after ester extraction, containing 8.32% methoxyl similar to the alkali lignins obtained prior to ester extraction. Contamination of the lignin appears to occur during the alkali treatment presumably by reaction between lignin and other substances soluble in the alkali. With cereal straw alkali lignin of high quality was obtained despite the presence of appreciable amounts of hemicellulose, and/

and it is highly probable that in the absence of nitrogenous material hemicelluloses do not condense with lignin in alkaline solution. With mature lucerne containing both nitrogenous material and hemicelluloses the alkali lignin was of low quality.

Material analysed	N% in ash-free lignin	MeO% in ash-free lignin	MeO% in corr. lignin
1. Alkali lignin from 2nd extraction (11B, Table A14)	3.06	7.64	9.44
2. Acid lignin from the pulp (TableA16)	2.01	8.64	9.89
3. Ester lignin from the pulp	0.85	12.52	13.47
4. Alkali lignin (from 1.25N NaOH) after ester lignin extraction (Table A14)	-	8.32	-

4.5. The quality of residues obtained during acid lignin determinations.

The behaviour of the 'soluble' fraction of the alkali lignin from seeds-hay resembled that of the various residues obtained during acid lignin determination and in Table A19 the analyses of some of these residues are shown. The resemblance of these residues to the acid lignin obtained by the modified procedure of Ellis et al, particularly in respect of methoxyl content, is apparent. With the exception of the precipitate obtained from conc. HCl washings, these residues did not exceed 7% of the ash-free acid lignin fraction, but they could nevertheless be responsible for variations in replicate determinations unless precautions were taken to avoid their loss during filtration and washing. All these residues gave deep colours with phenol reagent, even when the nitrogen content was low (seeds-hay and faeces from Timothy hay) indicating the presence of phenolic groups in the residue, probably of lignin origin.

(i) Humin precipitates from dilution of 72% H_2SO_4 filtrates.

Whether pepsin (Table A19, treatment No. 2) or trypsin (treatment No. 3) digestion was used in the pretreatment of seeds-hay, the amount of the precipitate was small, no doubt because the washing with conc./

conc. HCl removed much residue of similar character. Beside the compositional similarity to prepared acid lignin the reactions with phenol reagent and chlorine-Na_2SO_3 were like those of lignin. It was found later that if the conc. HCl extraction was omitted, this humin fraction increased greatly but the composition still resembled that of prepared acid lignins (Treatment No. 3 for faeces, Table A19). The same resemblance was also observed with young clover.

(ii) <u>The Residue from dilute acid hydrolysis.</u>

Both 5% HCl and 5% H_2SO_4 were used with seeds-hay, (Treatments 4a and 4b, Table A19) and the filtrates of the hydrolysates were at first clear but after standing produced residues with methoxyl contents similar to acid lignin, and giving positive reactions with phenol reagent and chlorine-Na_2SO_3. The nitrogen percentages were very much lower than in acid lignin. If the residue obtained after standing one night was filtered off, the clear filtrate yielded a further residue after standing for another day or after refluxing for one hour - the amount being much greater in the latter case. As it is unlikely (P147) that carbohydrates soluble in the dilute acid would resinify, and as the residues are of lignin character, it seems that

lignin/

lignin can be partly dissolved or highly dispersed by dilute acid hydrolysis (as was indicated by the loss of some alkali lignin during treatment with 72% H_2SO_4 followed (without drying) by final hydrolysis (Treatment 2.3 Table A15)) and that on long standing the dissolved or highly dispersed lignin forms larger aggregates.

Although the residue which settled down from the hydrolysates was of small magnitude, the fraction which remained soluble may have been far greater. It is therefore concluded that dilute acid hydrolysis should be omitted from the pretreatment because of the losses of lignin and changes in the character of the lignin which it produces. In testing the method of Armitage et al after 5% HCl hydrolysis, filtration and washing with water, the residue was washed with 0.25% Na_2CO_3 (the medium used in the subsequent trypsin digestion); this dissolved dark coloured substances which, after passing through the alkali lignin extraction procedure, gave an appreciable amount of lignin residue. When trypsin digestion with 0.25% Na_2CO_3 was carried out directly after ethanol-benzene extraction of seeds-hay, however, (without dilute acid hydrolysis) practically no loss of lignin occurred (P228 and Table A8). Dilute acid hydrolysis was also found to lead to the loss of

lignin/

lignin material by solution in the alcohol used to dehydrate the residue.

(iii) Residues from Alcohol washings.

After the pepsin-HCl digestion of plant material, only negligible amounts of residue dissolved in the alcohol washings, but there were noticeable amounts (about 4% of the acid lignin) after dilute acid hydrolysis. After treating the residue by the alkali lignin extraction procedure the methoxyl percentage was comparable with that of acid lignins, and positive reactions were obtained with phenol reagent and chlorine sodium sulphite.

With faeces it was found that after pepsin-HCl digestion, (Treatments 2, Table A19) noticeable amounts of residue dissolved in alcohol, and these were recovered by evaporation; when dissolved in alkali they gave dark solutions similar to lignin, and were precipitated by acid, and granulated in a manner similar to lignin, although the residue had a dirty appearance. The methoxyl percentage was lower than that in acid lignin. The amount which dissolved in alcohol was noticeable in the case of faeces but not with the food, possibly due to the greater amount of lignin present in the faeces (two to three times as great) or to some changes occurring in the alimentary tract.

(iv)/

(iv) Residues from conc. HCl washings.

The residues precipitated by diluting the conc. HCl washings of plant material were treated only with ethanol-benzene or with ethanol-benzene and acid pepsin and were found to be over 30% of the Ellis lignin, and of similar quality in respect of nitrogen and methoxyl contents. (Seeds-hay treatments 5 and 6, Table A19). The removal of soluble materials by pepsin digestion (about 20% of the original D.M.) did not change the quality of the residue. Positive reactions were given with both phenol reagent and chlorine-Na_2SO_3, and after passing through the alkali lignin extraction procedure, the residue gave an alkali lignin which was typical in respect of colour, and mode of precipitation and coagulation. It is concluded that the residue is lignin and should be included in any quantitative determination. The proposed method 1 was intended to determine only the lignin fraction insoluble in 72% H_2SO_4; as this fraction might be expected to be resistant to digestion, its determination is likely to serve a useful purpose, although in order to measure the whole lignin content of plant or other material, this method is clearly inadequate.

The general assumption that the lignin of feeding stuffs is insoluble in strong acids appears to be

incorrect/

incorrect; from the lignin nature of the various residues obtained it is clear that part of the lignin can dissolve in strong acids and this soluble fraction must be taken into consideration in any method intended to measure the total lignin content.

Summary :

Several preparations of alkali lignin were made from seeds-hay, young clover, oat straw, lucerne and faeces and their qualities studied and compared with those of acid lignins obtained from the same starting materials and also from some of the alkali pulps. Alkali lignin prepared from seeds-hay was subjected to detailed study including an examination of the behaviour towards the conditions used in acid lignin determination.

The main conclusions were as follows :-

(1) The modification of Bondi and Meyer's procedure for alkali lignin gave a purer product and was less tedious.

(2) The pretreatment used before extraction of alkali lignin had some effect on the quality of the lignin obtained.

(3) The alkali lignin partially dissolved in 72% H_2SO_4, and dissolved completely in conc. H_2SO_4 and in aceto-acetic ester in the presence of conc. HCl.

(4)/

(4) The alkali lignin soluble in 72% H_2SO_4 resembled the humin precipitates obtained during acid lignin determination which were found to originate from lignin, whilst the alkali lignin insoluble in 72% H_2SO_4 resembled the acid lignin fraction insoluble in 72% H_2SO_4.

(5) In the final stage of acid lignin determination filtration difficulties arose due to the humin lignin residue, which coagulated during the boiling with dilute acid. This final hydrolysis or washing with boiling dilute acid prevented appreciable losses of the humin fraction, whereas washing with water alone led to losses.

(6) Alkali lignin after treatment with 72% H_2SO_4 and drying was not affected by dilute acid hydrolysis, but vigorous hydrolysis reduced the nitrogen and methoxyl contents appreciably, although the remaining part of the lignin was relatively little affected in the hydrolysates.

(7) When alkali lignin was treated with 72% H_2SO_4 and then hydrolysed directly (without drying) with 3% H_2SO_4 it lost about 15% of its weight in two hours.

(8) The alkali lignin insoluble in 72% H_2SO_4 appeared to diminish the losses of the soluble humin lignin fraction when the latter was precipitated by dilution before filtration.

(9)/

(9) At least part of the nitrogenous material in lignin is of protein nature and the whole of it is probably a contaminant.

(10) When prepared from plant material of very low nitrogen content alkali lignin may serve as a reference substance, but the alkali lignin from other materials may be very impure.

(11) Acid lignin prepared from alkali pulp was of fairly constant quality, but was still contaminated with both nitrogenous and non-nitrogenous material, particularly the latter.

(12) The current opinion that lignin in immature plants is of low methoxyl content was not supported by the results obtained with young clover and immature pasture.

(13) The evidence (Doreé 1947, P367) that almost quantitative recovery of lignin methoxyl is obtained in the lignin fraction given by treatment with 72% H_2SO_4 was supported. Provided there are no losses in the humin lignin fraction the methoxyl not recovered in the acid lignin residue most probably belongs to non-lignin materials present in the plant material.

(14) The results indicated that combination of lignin with associates in the plant occurs during the alkali extraction procedure.

(15)/

(15) The assumption that the lignin of feeding stuffs is completely insoluble in strong acids appears to be incorrect and the soluble fraction needs to be considered in any quantitative determination.

(16) Dilute acid prehydrolysis removes part of the lignin and should be omitted.

(17) The proposed method 1 enables the determination only of the lignin fraction insoluble in 72% H_2SO_4, and this resistant fraction may prove to be resistant to digestion and so of biological significance.

(18) Methoxyl content was found to be the best indication of lignin purity.

CHAPTER V.

FACTORS AFFECTING THE DETERMINATION AND COMPOSITION OF ACID LIGNIN

The earlier experiments showed that even when prepared from alkali extracted material, acid lignin was contaminated and correction for crude protein by deducting N x 6.25, did not raise the methoxyl percentage to a level comparable with that of 'purer' lignin preparations. The current methods giving figures for ash-free or corrected (ash-free - N x 6.25) lignin could therefore only be regarded as unreliable and, in order to clarify the position, a study of the properties of acid lignin prepared under various conditions was necessary. The earlier work had shown the necessity to abandon dilute acid hydrolysis in the pretreatment, but the effect of final hydrolysis of the lignin residue, and of other factors, remained to be examined. Dilution of 72% H_2SO_4 extracts before filtration was necessary in order to obtain the whole lignin fraction.

5.1 Preparation of the plant material.

The materials used were seeds-hay sample 1 (oven dried), seeds-hay sample 2 (air dried) immature grass, young clover, and faeces from sheep fed on seeds-hay; separate portions of the faeces were dried at temperatures of 70°C and 105°C and a further portion was dehydrated with alcohol and dried at 50°C.

The/

The fresh young clover was chopped into pieces about ½ to 1 cm. in length and portions were then treated in different ways as follows :

(a) 150g. were washed several times with water on a cheese cloth fitted to a Buchner funnel, then digested for 24 hours with pepsin-HCl, further washed with water, dehydrated with alcohol and finally extracted with ethanol-benzene and dried at 50°C.

(b) 150g. were incubated for 24 hours at 40°C first with water, then with pepsin-HCl, and finally with trypsin-Na_2CO_3 and the residue was then treated as in (a) above.

(c) 25g. were dehydrated with alcohol in Soxhlet extraction thimbles, then extracted with ethanol-benzene and dried at 50°C.

(d) The remaining clover was oven dried at 105°C.

Samples (a) and (b) were easily handled, but for small samples the procedure used with sample (c) was found to be convenient and rapid. The nitrogen contents of samples prepared in these ways were lower than in the air dried clover.

The other fresh materials were soaked in 95% alcohol for several hours (sometimes overnight) so as to give a concentration of about 70% alcohol to diminish plant enzyme activity and facilitate drying; the/

the alcohol was then drained off and the samples dried for two days at 50°C. The fresh immature pasture grass was chopped before this treatment. Finally, after drying, all samples were finely milled.

Washing with water or incubating with water or pepsin-HCl at 40°C or soaking in cold alcohol is unlikely to remove lignin material from fresh plants, for the following reasons :-

(a) the absolute methoxyl content of ethanol-benzene extracted seeds-hay remained constant after digestion with pepsin HCl and even after trypsin-Na_2CO_3 digestion.

(b) Trypsin hydrolysates from young clover when acidified and passed through the alkali lignin extraction procedure produced a lignin fraction containing methoxyl to the extent of only 0.0033% of the original dry matter (Table A14); even if the young clover lignin contained only 11% methoxyl (as in the pulp acid lignin prepared), the extracted lignin would only amount to 0.036% of the original dry matter. Washing young clover with warm 0.5N NaOH only raised the extracted methoxyl to 0.0155% of the original dry matter, so losses arising from dilute alkali extraction of the fresh plant material may be regarded as negligible. Extraction with alcohol or dilute acid is likely to have even less effect than alkaline

solutions/

solutions and even Soxhlet extraction with warm alcohol or ethanol-benzene is not likely to remove more than traces of lignin. Although Cohen and [illegible] and Harris, [illegible] (1937) showed that water extracts from wood reacted with chlorine and sulphite, this might have been due to traces of lignin or possibly other material containing syringyl groups.

5.2 Methods.

Ethanol-benzene extraction and enzymatic digestion were (unless otherwise stated) as in the modified methods of Ellis et al (1946) and Armitage et al (1948) given in the Appendix (Page 12-17). Extraction with conc. HCl and the following main treatment with 72% H_2SO_4 were usually as in the proposed method 1 (P229) and, except when otherwise stated, the final hydrolysis by refluxing was for two hours with 3% H_2SO_4. The lignin residue was either hydrolysed directly, whilst still wet, or after being dried.

Losses of humin lignin during the final washing were avoided by using the technique found by Charmbury et al, (1945) to be satisfactory with humic acids prepared from coal. Washing first with 0.1N HCl prevented peptization in the subsequent water washing. The procedure finally adopted was to dilute the 72% H_2SO_4 solution 5-6 times, filter, wash with

0.1N HCl/

0.1N HCl until the washings were sulphate free, dry at 45°C and finally wash with water. The washing with HCl had the added advantage of being more efficient than water in removing sulphuric acid retained by the lignin residue.

5.3 Organic Solvent extraction.

5.3(i) The Extraction Process.

(a) Experiments with Seeds-hay, Sample 1 (oven dried).

In the Soxhlet apparatus used, the flasks were individually heated on water baths fitted with electric elements giving three different rates of heating. Although the normal method of operation was with the flasks seated above the hot water, by partially immersing the flasks it was possible to increase greatly the rate of extraction. It was possible to arrange four different syphoning rates, viz. once every three minutes, 10, 30 or 40 minutes. Usually 1g. samples were extracted and the dried residue weighed, the extractives including moisture being obtained by difference. In figure 5 (P. 36 Appx.) the first 6.61% of extractives would represent the moisture, the rest of the curve upward indicating the moisture-free extractives.

Extraction Time and Rate of Syphoning (Table A20).

The rate of extraction was always high at the beginning and decreased subsequently, the decrease being/

being most rapid when the frequency of syphoning was high. When syphoning occurred every three minutes the extractives increased rapidly during the first hour and then the rate declined but most of the extractives were removed in the first four hours; thereafter extractives continued to be removed at a slow, but more or less regular rate and the limit of extraction was not reached in 30 hours.

The difference in the yield of extractives with a high syphoning rate and with a low rate is large in the early stages but decreases greatly as the time of extraction is extended. (See Curves I and II, Fig. 5).

It is clear from the changes in slope of the curves (Fig. 5) that the material extracted was a mixture of at least two substances, one easily soluble and quickly extracted, and the other sparingly soluble, requiring more than 30 hours for extraction. The easily soluble fraction was extracted in about 1 hour when syphoning occurred every three minutes, but took about 15 hours when syphoning only occurred every thirty minutes. The very different extraction times recommended by different workers have probably been due to differences in syphoning rates, which have rarely been recorded.

A question of some practical importance in

connection/

connection with lignin determination is whether it is necessary to achieve complete extraction or whether extraction of the easily soluble fraction will suffice.

Size of Sample.

The extraction of large samples was necessary for some of the experiments requiring a bulk of homogeneous pretreated material and the results shown in Table A21 (for rapid extraction with a syphoning time of only 3 minutes) indicate that with larger samples, a longer period of extraction is preferable; after 4 hours, there was a noticeable difference between the yields of extractives from 1g. and 3.3g samples, although the difference may not have been significant.

Direct Reflux Extraction.

Direct reflux extraction of a 5g. sample was compared with Soxhlet extraction (with syphoning every 3 minutes); after one hour the extractives were 18.16% (reflux) and 18.50% (Soxhlet), indicating that direct refluxing is practically as efficient as Soxhlet extraction in the removal of the easily soluble material; after 4 hours, however, the extractives were 19.69% (reflux) and 21.98% (Soxhlet) showing that Soxhlet extraction was more efficient in removing the sparingly soluble extractives.

(b)/

(b) Experiments with seeds-hay, Sample 2 (air dried).

Using rapid (3 minutes) and medium (10 minutes) syphoning rates, the effect of extraction times varying from 15 minutes to 27½ hours was examined (Table A22 and Fig. 6)

In both extraction curves there is a sudden rise at the beginning corresponding to dehydration.

With the high rate of syphoning the results were similar to those with the previous hay sample, 4 hours being quite adequate for extraction of the easily soluble fraction. For the medium rate of syphoning the curve is very similar to that with the high syphoning/rate, but 4 hours extraction was only just sufficient to remove the easily soluble fraction, and 6 hours appeared to be preferable. As the time saved with the rapid syphoning rate was small it was found more convenient to operate the Soxhlet apparatus with a medium syphoning rate (10 minutes per cycle). At 25 hours extraction the two curves almost coincide and at 30 hours, extraction appears to be nearing completion. The seeds-hay sample 1 contained more sparingly soluble material than this sample 2, the extractives removed after the first 4 hours amounting to 4% in the first case and only 2% in the second.

From the experiments with the two seeds-hay

samples/

samples it was concluded that 4 hours with a high rate of syphoning was equivalent to 6 hours extraction with a medium rate of syphoning (10 minutes per cycle) for removal of the easily soluble extractives whilst the sparingly soluble fraction could be extracted in 30 hours (with either rate of syphoning) from some samples but not from others.

(c) Experiments with air dried young clover.

With ethanol-benzene syphoning onee every ten minutes (Table A23 & Fig. 7 , curve II) it was found that the rate of extraction was much lower than in the case of seeds-hay, the curve being similar to that for seeds-hay sample 1 when the syphoning rate was very low (once every thirty minutes). The limit of extraction had not been reached even after 48 hours, and the distinction between easily soluble and sparingly soluble fractions was not sharp. Nevertheless, as shown by the figures in the following table, (p.293) the absolute amounts of moisture-free extractives removed in any given time were much greater than in the case of seeds-hay.

Table (see over Page 293)

Owing to the larger amounts of the more soluble extractives present in young clover, something like 24 hours was necessary to remove them and after this time the rate of extraction ($\frac{\Delta E}{\Delta T}$) indicated by the

curve/

Extraction Time (syphoning every 10 min.)	Seeds-Hay Sample 2 (89.42% D.M.) Extractives (% of air dried sample)	Moisture-free extractives (% of D.M.)	Air Dried Young Clover Moisture-free Extractives (% of D.M.)
6 hours	21.12 x	11.81 +	16.38
24 hours	23.90	14.92	27.52

x obtained from curve Fig. 6

+ $(21.12 - 10.56) \; / \left(\frac{89.42}{100} \right) = 11.81$

curve was about 0.2% per hour, similar to the rate for seeds-hay sample 2 (air dried) after 6 hours extraction.

Ethanol-benzene only slowly removed chlorophyll, extraction being incomplete even after 20 hours. Alcohol alone was therefore examined as it is a better solvent for chlorophyll, giving complete extraction in 3 to 4 hours. The rate of extraction with 95% ethanol was greater than with ethanol-benzene, (Table A23) the curve I (Fig. 7) being similar to that for the ethanol-benzene extraction of seeds-hay. Fifteen hours extraction were necessary before the value of $\frac{\Delta B}{\Delta T}$ (obtained from curve I) became similar to the value for the ethanol-benzene extraction of clover after 24 hours (Curve II). After prolonged extraction (50 hours) the curves for ethanol benzene and 95% ethanol became almost parallel, the amounts of extractives being 30.6% and 33.2% respectively. (Similar figures were recorded by Thomas and Armstrong (1949) for 30 hours extraction of Lotus corniculatus (trefoil) and Plantago lanceolata (narrow leaved plantain) with ethanol benzene). Total extraction appeared to be almost complete after 40 hours with alcohol, but not after 50 hours with ethanol-benzene.

Although acetone extracted chlorophyll from clover/

clover moistened with a little water, it did not appear to be so efficient an extractant as ethanol or ethanol-benzene, and the nature of the extractives appeared to be different. With this solvent there was a sudden rise after a short period of extraction, followed by an almost linear curve, sloping upwards (Fig.1, curve III). The rate of extraction during the first 20 hours was lower than with ethanol-benzene or 95% ethanol and later the rate of extraction was greater than with the other solvents.

Petroleum ether was found to be the poorest solvent for removing green pigments and was far more specific than the others, extraction being nearly complete after 4 hours. When material which had been extracted for 9 hours with petroleum ether was further extracted for 14 hours with ethanol-benzene the amount of total extractives rose from 5.85 to 23.67% of the original D.M.

It seemed that a short period of extraction with 95% ethanol followed by further extraction with ethanol-benzene might have the advantage of removing the chlorophyll completely without prolonging the total extraction time. It was found that 6 hours extraction with ethanol followed by 12 hours with ethanol-benzene gave 31.86% extractives which represented almost complete extraction and was equi-

valent to/

to 40 hours extraction with alcohol alone or more than 50 hours with ethanol-benzene alone. Although the saving in time appeared to warrant the extra work of changing the solvents it was preferred to use a longer extraction time with ethanol-benzene alone in order to avoid any danger of loss of lignin.

5.3 (ii) Nature of the Extractives.

After extraction for some time brownish material was observed sticking to the side of the flask and by changing the flask and solvent after the first hour (syphoning every 3 minutes) it was possible to remove most of the pigments from seeds-hay, and from the latter extraction obtain the brown material relatively free from contamination. The extractives removed in the first hour contained fatty material and represented the easily extractable fraction; this fraction was insoluble in water and did not completely dissolve in 72% H_2SO_4. If not extracted, such material would clearly increase the lignin residue.

The brown material recovered from the later extraction appeared to be of low solubility in ethanol-benzene and therefore soon led to saturation and precipitation in the extraction flask. The dried material readily dissolved in cold water, dilute acid or alkaline solutions, but was only sparingly soluble

in/

in cold alcohol, though slightly more soluble in hot alcohol; when well stirred it dissolved fairly easily in 72% H_2SO_4 but it was insoluble in ether, benzene, acetone and chloroform.

It strongly reduced Fehling's solution and ammoniacal $AgNO_3$, gave a positive reaction in Molisch's test, and gave glucosazone with phenyl hydrazine. It appeared to be a soluble carbohydrate containing glucose and/or fructose (the only two hexoses known to be found in the free state in plants). The behaviour on long contact with 72% H_2SO_4 and the formation of humin substances and dilution resembled the behaviour of fructose rather than glucose (Chapter II).

It is concluded that this brown substance is not lignin and it was found that the pretreatment with pepsin-HCl solution extracted it more efficiently than the ethanol-benzene. When an air-dried seeds-hay sample was extracted 4 hours with ethanol-benzene, then digested with pepsin-HCl, dried and re-extracted with ethanol-benzene for a further 8 hours, only a negligible amount of material (3mg. = 0.3% of the original D.M.) was removed by the second extraction. It appears, therefore, that with material like hay extraction of the 'easily soluble' fraction is sufficient, provided it is followed by digestion with

pepsin/

pepsin-HCl solution. Either 4 hours extraction with ethanol-benzene syphoning every 3 minutes or 6 hours, syphoning every 10 minutes, is adequate.

Air dried young clover was extracted for 6 hours with ethanol-benzene (syphoning every 10 minutes) and for 4 hours with 95% ethanol, and in each case the extractives obtained after varying times were collected and examined. The ethanol-benzene extractives contained little chlorophyll and the residue strongly reduced Fehling's solution, but did not produce furfuraldehyde with HCl or react in the chlorine-Na_2SO_3 test; it dissolved almost completely in water (giving a slightly turbid solution), and was soluble in 72% H_2SO_4.

Although the alcohol extractives showed the same solubility in water and 72% H_2SO_4, they did not reduce Fehling's solution or react in Molisch's test. A positive reaction occurred with phenol reagent and with chlorine-sulphite, so it seemed desirable to avoid alcohol extraction in view of the possible removal of lignin-like material. The question still remained whether with young material like clover, a short extraction with ethanol-benzene was adequate as with seeds-hay, or whether more prolonged extraction was necessary.

5.3 (iii) Effect of prolonged extraction with organic solvents or water on quality of acid lignin.

Young air-dried clover was extracted with ethanol-benzene for 6 or 48 hours (syphoning every 10 minutes) and the effect of prolonged extraction on lignin quality was examined. Since some of the less readily soluble extractives might be removed by pepsin-digestion, or final hydrolysis with 3% H_2SO_4 for two hours, the effects of these treatments on the results obtained were also examined. The main treatment with 72% H_2SO_4 was in all cases carried out until the cellulose test gave a negative reaction and the lignin obtained contained both the fraction insoluble in 72% H_2SO_4 and the fraction soluble but precipitated by dilution.

(1) In treatments 1 and 3 (Table A24), omitting pepsin digestion and final hydrolysis, prolonged extraction with ethanol-benzene did not affect the lignin residue or the ash-free lignin, but reduced the corrected lignin from 8.22% to 6.53% owing to the increase of absolute nitrogen from 2.12% to 2.32%. The absolute methoxyl content was lowered from 0.472% to 0.400% by prolonged extraction, possibly due to the removal of associates containing methoxyl, which had condensed with the lignin, or to the removal of some lignin itself although the latter was unlikely as the ethanol-benzene extractives did not react in the chlorine - Na_2SO_3 test and had properties unlike lignin.

(2)/

(2) In treatments 2 and 4, corresponding to treatments 1 and 3 but including final hydrolysis of the dried lignin residue the absolute methoxyl content was again lower with the prolonged ethanol-benzene extraction, the difference for treatments 2 and 4 being similar to the difference for treatments 1 and 3, but the actual values were lower than when final hydrolysis was omitted. This may have been due to the removal of methoxyl-containing contaminants or of part of the lignin during final hydrolysis which also removed considerable amounts of nitrogenous material, (about 66%) and lowered the lignin residue by about 50% in each case. After N correction, however, the differences between treatments 1 and 2 and between 3 and 4 were much less, indicating that perhaps the majority of the nitrogenous materials were of protein origin, hydrolysable by 3% H_2SO_4.

(3) When pepsin digestion was introduced in treatments 5 and 6 (Table A24) the absolute methoxyl contents obtained were very similar, indicating that so long as pepsin digestion is used, prolonged ethanol-benzene digestion is unnecessary. The absolute methoxyl content is the best index of the amount of lignin present in the crude lignin fraction, and as it is very unlikely that pepsin digestion removed any lignin, it appears (by comparison with treatments 1 and 3) that this treatment is efficient in removing methoxyl/

methoxyl-containing contaminants. Although it appears that the effect of prolonged ethanol-benzene extraction when pepsin digestion is omitted (Treatment 3) is also to remove methoxyl-containing contaminants, even an additional 42 hours extraction was less efficient than pepsin digestion. Pepsin digestion of clover (See Table A25) removed from the ethanol-benzene residue 40.71% of the original dry matter, whereas prolonging the ethanol-benzene extraction from 6 hours to 42 hours removed only 14.2% of the original dry matter. Moreover, pepsin digestion after 6 hours ethanol-benzene extraction removed 50% of the absolute methoxyl in the ethanol-benzene residue (from 1.725% to 0.858%) (methoxyl-containing contaminants) and 77% of the absolute nitrogen (from 3.38% to 0.792%) as well as 77% of the nitrogen of the lignin residue. (See treatments 1 and 6, Table A24). This showed that the greater part of the N in the lignin residue is of protein origin and can be enzymatically removed, and that it is satisfactory to use a short extraction with ethanol-benzene, followed by pepsin digestion.

It should also be noted that when prolonged ethanol-benzene or alcohol extraction was used without final hydrolysis (Treatments 3 and 5, Table A24, Treatment/

Treatment 3, Table A26) the lignin residue had a relatively high absolute nitrogen content. From treatments 5 and 6 of Table A24, it appears that prolonged extraction with ethanol-benzene changes the nature of the nitrogenous material making it more resistant to pepsin digestion or hydrolysis with 72% H_2SO_4. In treatment 7 when final hydrolysis was applied directly to the wet lignin residue, a noticeable reduction in absolute methoxyl was obtained, (compared with treatment 5) most probably due to loss of lignin itself.

In all cases except treatment 7 (Table A24) the absolute methoxyl contents were higher than that in the lignin obtained by the modified Ellis et al procedure, probably because of losses of lignin in the Ellis method as indicated previously.

(4) Prolonged extraction with alcohol, or acetone, or 6 hours ethanol-benzene extraction followed by incubation in water overnight (Table A26) had the same effect on the absolute methoxyl content of the lignin residue as prolonged ethanol-benzene extraction (Treatments 3 and 4, Table A24). Before final hydrolysis, the absolute methoxyl content was about 0.39% (0.381% to 0.407%) and after hydrolysis, about 0.31% (0.296% to 0.326%), the decrease being highly significant./

significant. Pepsin digestion, however, was still more efficient; when used without final hydrolysis 0.345% absolute methoxyl was obtained (Treatment 6, Table A24) but when the dry lignin residue was hydrolysed (Treatment 8) 0.312% absolute methoxyl was obtained. The reduction in absolute methoxyl content produced by the final hydrolysis was probably not significant in this case, although when less efficient pretreatment was used (omitting pepsin digestion and using prolonged extraction with ethanol-benzene, alcohol, or acetone, or incubation with water) the final hydrolysis did reduce the absolute methoxyl content very significantly, probably because of contamination with easily hydrolysable associates containing methoxyl. From these and previous results it seems that associates present in the lignin fraction are mostly affected by final hydrolysis, but lignin itself may remain unaffected. It was found previously (P258) that alkali lignin after treatment with 72% H_2SO_4 was not affected by final hydrolysis with 3% H_2SO_4, provided it was dried before hydrolysis and Heuser and Schmelz, 1920 found that heating lignin with 5% HCl in a sealed tube at 150-160°C for 3 - 5 hours produced no loss of methoxyl.

(5) The main conclusion is that provided pepsin

digestion/

digestion is used, there is no necessity for prolonged extraction with ethanol benzene, and for a general treatment 4 hours extraction with ethanol-benzene, syphoning every 3 minutes, or 6 hours, syphoning every 10 minutes, is satisfactory. There seems to be no objection to ethanol-benzene extraction, and no advantage in the use of other organic solvents, so long as pepsin digestion is included in the pretreatment. Pepsin digestion is more efficient in removing associates containing methoxyl as well as much nitrogenous material and most of the nitrogenous material in the lignin residue appears to be of protein origin.

The final hydrolysis might not be necessary if pepsin digestion were used as the absolute methoxyl content in the lignin residue did not seem to be significantly reduced, provided the lignin was carefully filtered and dried prior to the hydrolysis.

5.4 Effect of Dilution of 72% H_2SO_4 on acid lignin quality.

5.4 (i) Experiments with air-dried seeds-hay.

In the proposed method 1 the 72% H_2SO_4 extract is filtered without dilution and comparison with the modified methods of Armitage et al, (1948) and Ellis et al, (1949) (Table A27) shows that avoiding dilution gives lower yields of ash-free and corrected lignin.

(a)/

(a) Effect of Dilution of 72% H_2SO_4 after using conc. HCl extraction.

Comparison of treatments 3 and 6 (Table A27) shows that dilution of the 72% H_2SO_4 before filtration gave a slight but probably insignificant increase in ash-free lignin and corrected lignin. After extraction with conc. HCl the humin precipitate from dilution of the 72% H_2SO_4 was probably extremely small. The quality of the lignin from treatment 6 was slightly poorer than that from the standard method (Treatment 1) and the absolute methoxyl content was much lower than in either the Ellis or the Armitage lignin, indicating that even when the 72% H_2SO_4 was diluted before filtration, proposed method 1 gave a lignin fraction containing less absolute lignin than the current methods. The final residue from treatment 6 was washed with water in a sintered glass crucible, and some loss of humin fraction was observed, which probably accounts for the slightly higher yield of absolute methoxyl in treatment 8; the final hydrolysis had been expected to reduce the absolute methoxyl.

The omission of pepsin digestion (Treatment 7) gave lignin with a nitrogen content three times as great as that from Treatment 6, but the absolute methoxyl content was slightly higher, though

probably/

probably not significantly so as it was similar to that of the lignin from treatment 8. This suggested that pepsin digestion might be omitted with this type of plant material if the result could be based on absolute methoxyl content rather than expressed as ash-free or corrected lignin.

The yields of absolute methoxyl from Treatments 6, 7 and 8 (Table A27) were more than three times the methoxyl in the acid lignin obtained from the alkali pulp, (Treatment 9) indicating that the alkali extraction of seeds-hay (0.5 N-NaoH at 85°C) removed a considerable proportion of the lignin fraction which was insoluble in 72% H_2SO_4.

(b) Effect of dilution of 72% H_2SO_4, omitting conc. HCl extraction.

The omission of conc. HCl extraction in treatment 10 (Table A27) surprisingly gave a lignin of quality similar to that obtained by the standard Ellis method,(Treatment 1) although the yields of absolute methoxyl, ash-free lignin and corrected lignin were about two-thirds greater with Treatment 10 than with Treatment 1 and more than twice as great as from Treatments 6 and 8. It was concluded that the humin fraction formed more than 50% of the total lignin, and that great losses of this fraction had occurred in the standard and proposed methods.

With/

With seeds-hay the omission of dilute acid hydrolysis from both the pretreatment and final treatment gave a lignin with similar quality to that obtained by the modified method of Ellis et al.

In Treatments 11 and 12 the wet lignin residues were finally hydrolysed with 3% H_2SO_4 for two hours and this gave a reduction in ash-free lignin, corrected lignin and absolute methoxyl although the percentage of methoxyl was slightly higher than in Treatments 1 and 10, indicating that final hydrolysis removed substances other than lignin, more rapidly than lignin itself.

In Treatment 13 pepsin digestion was omitted, but final hydrolysis included and the lignin had a methoxyl percentage similar to that from Treatment 10 although the N content was considerably greater. The absolute methoxyl content had been expected to be at least equal to that from Treatments 11 and 12, but was slightly (though probably not significantly) lower. ~~The colloidal natu~~when the final hydrolysis was applied to the wet residue and the final washing was with water, as in this experiment the humin fraction was inevitably colloidal, leading to some losses.

(c) Reaction of lignin residues in Furfural and Fehling's tests.

The Furfural test was made in two ways :

(i)/

(i) addition of solid phloroglucinol to the lignin residue, followed by boiling with 12% HCl.

(ii) boiling the lignin residue in 12% HCl and detecting the released furfuraldehyde with a piece of filter paper moistened with aniline.

The latter test was not very sensitive as small amounts of furfuraldehyde could escape without giving the red colour with aniline. The former test was in several cases rendered indefinite by the brown solution formed by the action of 12% HCl alone on the lignin residue, but this difficulty was overcome by cooling the reaction mixture, filtering through Whatman No50 paper and washing with water when the red precipitate of furfural phloroglucide was easily detected.

The modified Ellis and Armitage methods gave lignin which reacted positively to both furfural and Fehling's tests. The furfural reaction was only negative in cases where conc. HCl washing was used in the pretreatment (Treatments 3 to 8, Table A27), and reduction of Fehling's solution occurred in all cases except treatment 4. Even in treatment 4 there may have been some slight reduction masked by the lignin residue.

Conclusions.

Proposed method No. 1 produces a lower lignin yield containing less absolute methoxyl than the/

the current methods. Omission of the conc. HCl treatment and dilution of the 72% H_2SO_4 extract is necessary in order to obtain the total lignin. Final hydrolysis when applied to the wet lignin residue seems to remove a fraction of the lignin and so may be undesirable. Figures for absolute methoxyl content indicate that the lignin fractions obtained by current methods do not contain the whole of the plant lignin. Conc. HCl washing removes furfural-producing substances which appear to be invariably present when HCl extraction is omitted. Reduction of Fehling's solution seems to indicate the presence of carbohydrates contaminants, although reduction may possibly be due to reducing groups in the lignin itself.

5.4 (ii) Experiments with air-dried young clover.

(1) Proposed method 1 (Treatment 3, Table A28) or the modification substituting trypsin for pepsin digestion (Treatment 5) produced a higher yield of ash-free lignin than the standard method (Treatment 1) but there was little difference in the corrected lignin. Final hydrolysis (Treatment 4) reduced both the ash-free and corrected lignin (Compared with Treatment 3) although the reduction was less when trypsin digestion was substituted for pepsin digestion. (Treatment 6). The reduction was no doubt due to

the/

the large amount of nitrogenous materials present in the lignin from proposed method 1 (even after final hydrolysis the nitrogen percentage was almost double that of the Ellis lignin) and it was obvious that for plant material of high nitrogen content enzymatic digestion removed only small amounts compared with enzymatic digestion plus prehydrolysis as in the Ellis and Armitage methods. Even these latter methods (Treatments 1 and 2, Table A28) gave lignin contaminated with 2.39% to 3.99% of nitrogen. With the addition of final hydrolysis proposed method 1, and the modification using trypsin, (Treatments 4 and 6) gave yields of absolute methoxyl lower than the Ellis method (standard), but similar to the Armitage method and (surprisingly) to the acid lignin from the alkali pulp. Thus with young clover the lignin fraction insoluble in 72% H_2SO_4 was also unaffected by alkali extraction, a result completely different from that obtained with seeds-hay.

(2) When conc. HCl extraction was avoided, and the 72% H_2SO_4 was diluted before filtration (Treatments 8 and 9, Table A28) the yields of absolute methoxyl, ash-free lignin and corrected lignin were considerably greater than in the Ellis method and the quality of the lignin was somewhat poorer. When the lignin fraction/

fraction from Treatment 8 was washed with 0.1N HCl, dried and then hydrolysed for two hours with 3% H_2SO_4 (Treatment 10), the decrease in absolute methoxyl was small and possibly insignificant, as indicated with previous results (Section 3, P302). The absolute methoxyl was still greater in the Ellis lignin, although it appeared that losses of lignin in the Ellis method were relatively small in contrast with the results obtained with seeds-hay.

(3) The effect of avoiding enzymatic digestion but diluting the 72% H_2SO_4 is shown by treatment No. 1 in Table A29. The yield of ash-free lignin was very high and it contained considerable amounts of nitrogenous material and had a low methoxyl percentage, although the absolute amounts of methoxyl was relatively high. As the absolute methoxyl in the ethanol-benzene extracted clover was considerably higher than in the product after both ethanol-benzene extraction and pepsin-HCl digestion, (Treatments 2 and 3, Table A25) the difference was attributed to removal of methoxyl-containing contaminants, and it seems very likely that the relatively large amount of absolute methoxyl in the lignin obtained by treatment 1 of Table A29 was also due to the presence of contaminants containing methoxyl.

5.5. Effect of hydrolysis on very impure lignin from young clover.

A/

A large sample of air-dried clover (equivalent to 7g. original dry matter) was extracted with ethanol-benzene for 6 hours then treated with 72% H_2SO_4 below 20°C until the cellulose reaction was negative, diluted approximately 5 times, filtered through Whatman No. 50 paper, transferred to a sintered glass crucible, washed with 0.1N HCl, dried at low temperature, rewashed with water and finally dried, giving a yield of 1.5133g. crude lignin. Portions of this were subjected to varying degrees of hydrolysis as shown in Table A29 and Fig. 8, A and B.

(1) One hour hydrolysis with 3% H_2SO_4 produced a marked decrease in ash-free lignin owing to the removal of about 60% of the nitrogenous material; corrected lignin decreased about 18% and absolute methoxyl about 14%, indicating that the methoxyl was least affected, and it is probable that the methoxyl lost belonged to contaminants rather than lignin. When hydrolysis was prolonged to 5 hours the further loss of nitrogenous material was still considerable, but subsequently the rate of removal declined and 10 hours hydrolysis removed about 89% of the nitrogen, although the ash-free lignin still contained 3.71% nitrogen. The yield of ash-free lignin showed a similar reduction but the fall in corrected lignin was/

was relatively less and practically complete in 5 hours, indicating that after that time there was very little removal of non-nitrogenous material.

The methoxyl percentage in the corrected lignin was a maximum after 5 hours hydrolysis (Fig. 8 BI) but the subsequent decline does not necessarily indicate demethoxylation of lignin as removal of contaminants containing methoxyl might be occurring. After 10 hours/hydrolysis/the absolute methoxyl was 0.322% and similar figures were obtained previously, using different procedures, e.g. 0.345% before hydrolysis and 0.312% after 2 hours hydrolysis of the dry lignin when pepsin digestion was used in the pretreatment (Treatments 6 and 8, Table A24) and 0.311% to 0.326% when prolonged organic solvent extraction was followed by 2 hours hydrolysis (with 3% H_2SO_4) of the dry residue. (Treatment 4, Table A24, and Treatments 2 and 4, Table A26). The modified Ellis procedure gave 0.273% absolute methoxyl, (Table A28) and it is highly probable that a fraction of the lignin was lost in that procedure. Perhaps the figure of 0.345% (Treatment 8, Table A28) obtained after pepsin digestion (removing considerable amounts of methoxyl-containing associates - see Table A25) was a fair approximation to the absolute methoxyl content of the lignin, and this is supported

by/

by the fact that when precautions were taken in filtering and drying, 2 hours final hydrolysis with 3% H_2SO_4 only reduced the absolute methoxyl to 0.312%.

From the results obtained with air-dried clover it seemed desirable to use pepsin digestion in order to obviate prolonged organic solvent extraction, and give a lignin fraction less contaminated with methoxyl-containing associates, probably rendering final hydrolysis unnecessary.

(2) Prolonged hydrolysis of the highly contaminated lignin fraction, finally produced a lignin residue which, though of improved quality, was still inferior to the acid lignin obtained by the standard method (modified Ellis) and the acid lignin obtained from the pulp. (the latter being the best acid lignin obtained from young clover). Even after correction for crude protein, the lignin fraction remaining was still high and appeared to contain non-nitrogenous contaminants resistant to dilute acid hydrolysis, since the percentage was only almost half of that in the acid lignin from the pulp. (Treatment 7, Table A28).

(3) After vigorous hydrolysis for 24 hours (Treatment 5, Table A29) the corrected lignin was similar to that obtained after 10 hours mild hydrolysis, indicating that the contaminants remaining after 10 hours mild hydrolysis are in fact extremely resistant

and/

and difficult to remove. The vigorous hydrolysis removed 96% of the nitrogen so the contaminants remaining were obviously non-nitrogenous. Demethoxylation probably occurred during the vigorous hydrolysis since comparison of treatments 4 and 5 in Table A29 shows a much greater decrease in absolute methoxyl than in lignin. The filtered hydrolysate gave a positive reaction in the test for α-amino acids and the extent of hydrolysis of the nitrogenous material was typical of protein and similar to the results obtained in the vigorous hydrolysis of alkali lignin from seeds-hay (ChapterIV).

Summary.

From a study of the quality of acid lignin obtained from seeds-hay and clover by various procedures the following conclusions were drawn :-

(1) Pepsin digestion following a short ethanol-benzene extraction appears to be the most satisfactory general pretreatment.

(2) There are two fractions of lignin requiring consideration, the fraction insoluble in 72% H_2SO_4 and the total lignin. The former may be obtained by proposed method 1, and the latter by omitting conc. HCl extraction, and diluting the 72% H2SO4 before filtration. With both fractions the absolute methoxyl/

methoxyl content appears to be the best indication of the absolute lignin present, figures for ash-free or corrected lignin being less reliable because of persisting contamination.

(3) Final hydrolysis may not be necessary if pepsin digestion is used. If lignin is obtained without pepsin digestion it is necessary to distinguish between methoxyl belonging to associates (removed by mild hydrolysis) and methoxyl belonging to lignin, (resistant to mild hydrolysis) particularly in the case of young plants like clover, with a high proportion of methoxyl-containing associates in the ethanol-benzene extracted material.

5.6 Other factors affecting the Quality of Acid Lignin.

5.6 (i) Effect of prolonged enzymatic pretreatment.

Young green clover was pretreated in a manner simulating the digestive process of the ruminant (treatment 1, Table A30). The chopped material was incubated 24 hours at 40°C with water (similar to rumen) then for 24 hours with acid pepsin (as in abomasum) and finally for 24 hours with alkaline trypsin (as in small intestine) in order to remove most of the nitrogenous material and methoxyl-containing associates. It was then extracted with ethanol-benzene, dried at 50°C, treated with 72% H_2SO_4, diluting before filtration and the wet lignin residue finally hydrolysed for 2 hours with 3% H_2SO_4. The quality of the lignin obtained was no better than that of the lignin from treatment 2 (Table A30) where the green plant material was only washed with water and incubated with acid pepsin prior to ethanol-benzene extraction and digestion with 72% H_2SO_4. The absolute methoxyl contents were of the same order. Thus prolonged enzymatic pretreatment does not produce a high quality acid lignin, and trypsin digestion does not reduce lignin yield (as indicated by the figures for absolute methoxyl) in confirmation of previous results (Treatments Nos.8,9 Table A28).

5.6 (ii)/

5.6 (ii) Effect of omitting pepsin-digestion in the pretreatment of faecal material.

Using the two pretreatments indicated in Table A31 the effect of omitting pepsin digestion in the pretreatment of faeces from sheep fedpon seeds-hay, was examined. In each case the pretreated faeces were digested with 72% H_2SO_4 until the cellulose reaction was negative, then dilution and filtration was followed by 2 hours final hydrolysis with 3% H_2SO_4.

The lignin fraction obtained was of practically the same quantity and quality whether pepsin digestion was used or omitted although previous experiments with seeds-hay using similar procedures, (Treatments 11, 12, 13, Table A27) showed that enzymatic digestion lowered the nitrogen content and improved the quality of the lignin. Thus in the case of faecal material it is the biological pretreatment provided by the digestive process which renders further enzymatic digestion unnecessary.

5.6 (iii) Effect of Temperature of Drying

(a) The Drying of pretreated young clover

Treatments 2 and 3 of Table A30 show that after the pretreatment of young clover with pepsin and ethanol-benzene, drying at 105°C for 3 hours or at 50°C before subjection to the main treatment gave virtually the same final yield and quality of lignin with/

with the same absolute methoxyl content. From this it may be predicted that the temperature of drying faecal material derived from green young fodder will have little or no effect on the lignin yield or quality, since the process of digestion will be comparable with the pretreatment used for young clover.

(b) The Initial drying of Fresh Clover and Faeces

Oven drying at 105°C and at 70°C was compared with alcohol dehydration followed by drying at 50°C. The materials oven dried at 105°C or 70°C were milled and Soxhlet extracted with ethanol-benzene for 4 hours whilst the other fresh samples were dehydrated with alcohol in extraction thimbles and then extracted with ethanol-benzene for 4 hours, dried at 50°C and milled. All the milled samples were then digested with pepsin-HCl for 24 hours at 40°C followed by digestion with 72% H_2SO_4 at room temperature (below 20°C) until the cellulose reaction was negative. In some cases (as indicated in Table A52) 2 hours final hydrolysis with 3% H_2SO_4 was also employed.

(1) With young fresh clover - When final hydrolysis was carried out the yield of lignin residue obtained after drying at 105°C was much greater than after alcohol dehydration and drying at 50°C (Table A52). The ash-free lignin in the former case contained

far/

far more nitrogen than in the latter, and the absolute methoxyl was about 20% greater. This indicated clearly that initial oven drying at 105°C should be avoided, particularly if the basis of comparison is ash-free lignin or corrected lignin. Oven drying seemed to affect the physical condition of the tissue, particularly the protein materials, so that the lignin residue was highly contaminated.

When final hydrolysis was omitted (treatments 4 and 5, Table A32), the results were in both cases higher than when hydrolysis was included and the ash-free lignin, corrected lignin and absolute nitrogen were much higher after drying at 105°C than after alcohol dehydration and low temperature drying. The absolute methoxyl content after high temperature drying was 53% greater than after low temperature treatment; this was probably due to the presence of methoxyl-containing associates since the absolute methoxyl content was reduced from 0.480% (treatment 4) to 0.332% (treatment 1) by final hydrolysis. In the two cases of low temperature drying, however, the absolute methoxyl contents were much closer, being 0.310% without final hydrolysis (treatment 5) and 0.273% with hydrolysis (treatment 3) indicating that the great loss in methoxyl produced by final hydrolysis in the case of high temperature drying, was probably due to easily removed contaminants containing/

containing methoxyl.

Results based only on ash-free or corrected lignin would thus have given a misleading picture indicating a necessity for low temperature drying and final hydrolysis to get the lowest yield (as in treatment 3). The absolute yields of methoxyl from treatments 1, 3 and 5 however, are all of the same order (0.332, 0.273 and 0.311); perhaps when low temperature drying is used, final hydrolysis becomes unnecessary, whilst high temperature drying may be permissible provided final hydrolysis is carried out to remove labile methoxyl.

(2) The results with faeces from sheep fed on seeds hay (treatments 1, 2, 3 Table A32) ~~The results~~ show that the yield and quality of lignin were unaffected by the drying temperature although a noticeable decrease in nitrogen content appeared to be associated with drying at 105°C. The absolute methoxyl contents were very similar.

It was also noted in these experiments that the amount of ethanol-benzene extractives removed from alcohol dehydrated clover, dried at 105°C, whereas was about twice as great as from clover with the faeces, the amounts of extractives in the two cases were almost the same. The inefficient extraction of the oven dried clover was probably a factor involved in the lower quality of the lignin obtained.

The/se

These results show clearly why a very high digestibility of lignin was indicated by experiments with young plants when a procedure such as that of Kalb (1932) was used (e.g. Bondi and Meyer, 1943) and both faeces and fodder oven dried. The drying would produce a great apparent increase (possibly three-fold) in the food lignin, while the biological pretreatment of the faeces (by the animal) would lower the faecal lignin.

5.7 Influence of pectic and protein materials on acid lignin quality.

5.7 (i) Pectic material

(1) It was found previously (Chapter III) that B.D.H. Apple pectin did not form insoluble products in 72% H_2SO_4 even after 5 days contact or after dilution (38 vols. H_2O) and final hydrolysis. This experiment was repeated and the results confirmed, but when mixed with 72% H_2SO_4 pectin formed lumps which took about three hours to dissolve.

(2) During the preparation of a pretreated sample by the modified method of Armitage et al (1948) some pectic material was identified in the precipitate obtained by acidifying the trypsin hydrolysate; it was extracted by ammonium oxalate, precipitated by alcohol and formed a gel with calcium salts. The pectic residue obtained resisted hydrolysis in 5% H_2SO_4 for over 8 hours. The same result was obtained with/

with apple pectin but in this case the acid hydrolysate was turbid, containing the pectin in a dispersed (colloidal) form. According to Hirst and Jones, (1946) pectic acid is extraordinarily stable towards acid hydrolysis and this was also confirmed by refluxing apple pectin with 12% HCl, 5N.HCl and 5N.H_2SO_4; furfuraldehyde was released but a bulky brown residue remained.

(3) It was concluded from these results that at least the insoluble pectins found in plants (as calcium or magnesium salts or combined with cellulose) would remain after pretreatment, and that therefore it was advisable to increase the time of contact with 72% H_2SO_4 over 2 hours to ensure solution of the pectic material. The further question arose whether (using pretreatment without dilute acid hydrolysis) the lignin residue might condense with methoxyl from pectic material, during the strong acid treatment and so become contaminated.

(4) Quantitative experiments were carried out with alkali pulp from seeds-hay (containing a minimum of interfering substances) acid lignin being prepared from it with and without the addition of apple pectin during the strong acid digestion (Treatments 1 and 2 Table A33). The yield and quality of the acid lignins obtained were practically the same. The recovery in the lignin of the total methoxyl originally present in the mixture of pulp and apple pectin, was much/

much lower (8.53%) than in the case of the pulp alone (64.8%) but the absolute methoxyl contents of the acid lignins were alike (0.169% and 0.173%) indicating that none of the pectin methoxyl was recovered in the acid lignin.

If the pectin methoxyl in the starting material is ignored, the recovery of pulp methoxyl in the lignin fraction (Treatment 2) becomes 63.0%.

It was concluded therefore that no interference from pectic material was likely to occur in acid lignin determination. During this experiment it was observed that the addition of a few drops of conc. HCl to the 72% H_2SO_4 greatly reduced the lumping of the pectin, and facilitated its solution.

5.7 (ii) Proteins

(1) Previous tests (Chapter III) showed that gelatin did not produce any precipitate in 72% H_2SO_4 even after long contact and dilution. When the experiment was repeated with 180mg of the plant protein gluten (B.D.H. containing 14.32% N and 0.3% methoxyl) and 20 ml 72% H_2SO_4 it was found that a fraction of the gluten remained insoluble after 3 hours, though complete solution, giving a purple solution, occurred after 17 hours contact. Portions of the acid filtrate after 2 hours and 3 hours contact were diluted with 38 vols of water and turbidity occurred, followed by the separation of a clean white precipitate which/

which after 2 hours boiling disappeared leaving the solution almost clear. Dilution after 17 hours and 22 hours contact with 72% H_2SO_4 (when all the protein had dissolved) produced only slight turbidity.

(2) The effect of 72% H_2SO_4 on gluten was quantitatively studied under conditions similar to those of lignin determination. In treatment No.1 (Table A34) it was found that after 3 hours contact with 72% H_2SO_4, followed by dilution, filtration and finally 2 hours refluxing with 3% H_2SO_4, a fraction of the protein remained insoluble but was only partly retained by a sintered glass crucible of pore porosity 3, the filtrate being turbid; refiltration through a Gooch crucible enabled a further amount to be recovered but the final filtrate was opalescent rather than transparent. The total recovery of the protein was only 5.87%.

In treatment No.2, the final hydrolysis was omitted and the recovery of protein retained by a sintered glass crucible was 40.5%. The percentage nitrogen in this recovered protein was 14.6% lower than in the original gluten, increasing the nitrogen conversion factor (100/%N) from 6.98 to 8.18.

In treatment No.4, 4.5 hours contact was used without final hydrolysis and even though filtered through a Gooch crucible the recovery of protein was lower than in treatment 2, showing that longer contact reduces/

reduces the insoluble fraction.

Treatment No.3 was similar to No.4 with the addition of final hydrolysis, and the yield of insoluble protein (12.1%) was lower than in No.4 (27.2%) but higher than in treatment No.1, although this may have been due to filtration losses in treatment No.1 as the filtrates of the protein hydrolysates appeared colloidal in nature.

These results indicated that plant proteins remaining in a pretreated plant sample would be partly insoluble in 72% H_2SO_4 and the subsequent hydrolysis and so lead to contamination of the lignin fraction. It was also to be expected that in the case of the plant material the recovery of protein material in the lignin would be greater, as the physical conditions would be less favourable to the solution of proteins in the 72% H_2SO_4 and some of the soluble proteins might even react with the lignin itself. That this was the case with seeds-hay and young clover is indicated by the results in Table A35 obtained with various pretreated samples and Sowden and De Long (1949b) have also reported a recovery in lignin of 70 to 76% of the N in pretreated plant material. The higher N conversion factor for the protein after treatment with 72% H_2SO_4 indicated changes in the nature of the protein, and it seemed that the protein fraction insoluble in the acid might contain

contain a higher proportion of aromatic amino acids which have high N conversion factors (7.29, 11.80, 12.94 for tryptophane, phenylalanine and tyrosine respectively).

(3) The effect of the addition of gluten to alkali pulp during acid lignin determination was examined in treatments 6, 7 and 8 of Table A33. In treatment 6 the addition of gluten increased the lignin yield from 1.64 (treatment 5) to 3.7; the nitrogen content greatly increasedd, the methoxyl percentage was lowered, but the absolute methoxyl was relatively unchanged. After vigorous hydrolysis of the residue (treatment 7) the lignin fraction and N content decreased but remained higher than for the control treatment 5 (with gluten omitted) Demethoxylation occurred as only 43.9% of the methoxyl present in the residue from treatment 6 was recovered. Thus even after vigorous hydrolysis, the lignin fraction remained contaminated with associates of non-protein origin which had probably condensed with the protein remaining in the lignin fraction. In other words the presence of protein material appeared to increase the contamination of lignin with carbohydrates. The great rise in the N conversion factor calculated by dividing the increase in ash-free lignin by the increase in nitrogen confirmed this view. On the basis of the nitrogen recovered, vigorous hydrolysis removed/

removed 88% of the protein which was similar to the amount of nitrogenous material hydrolysed in the treatment of acid lignin from young clover and alkali lignin from seeds-hay (Chapter V , Section 5 and Chapter V, P285) supporting the belief that the nitrogenous material in lignin is largely protein.

In treatment No.8 the contact time was increased to 24 hours and the ash-free lignin was lower than with short contact (treatment 6) but the corrected lignins were of the same order, indicating that the higher ash-free lignin after short contact was due to the presence of more protein material. The use of the usual N conversion factor of 6.25 seemed to be justified. The absolute methoxyl content in treatment 8 was practically the same as in control treatment No.5, and a little lower than in No.6, where the contact with 72% H_2SO_4 was for a shorter period.

It is obvious that except in the case of vigorous hydrolysis the absolute methoxyl in the lignin residue was the least affected. The recovery of nitrogen in the lignin was of the same order as that obtained using only pure gluten (Table A34) and it seemed highly probable that most or all the nitrogenous material recovered in the lignin fraction was due to its insolubility in 72% H_2SO_4, and during the final hydrolysis. Retention of protein in acid lignin due to condensation with the lignin seemed to be slight

or/

or non-existent. The recovery of protein in acid lignin from pretreated plant material (using final hydrolysis) was, however, found to be higher (Table A35) than the recovery of pure proteins added to alkali pulp, possibly due to the physical state in the intact plant where the protein may be enveloped by the insoluble lignin and some sort of linkage between protein and lignin may also exist. In the case of seeds-hay the recovery of protein was higher than in the case of young clover, but this may be the result of a larger amount of absolute lignin, relative to protein.

5.7 (iii) Effect of adding both pectin and protein

The effect of casein in the presence of pectin was examined in treatments 3 and 4 of Table A33 and the results were similar to the case when gluten was used alone; it appeared that pectin in the presence of protein had no effect on lignin yield and quality.

It should be noted that when the lignin residue from treatment No.3 was dried and then hydrolysed for 2 hours with 3% H_2SO_4 (treatment 4) the recovery of methoxyl after hydrolysis was 100% confirming the view that the methoxyl belongs to the lignin and is not affected by dilute acid hydrolysis.

5.8 General conclusions and suggestions for scheme of analysis

(1) Any acid lignin determination in feeding-stuffs based on ash-free lignin or corrected lignin is/

is unreliable owing to the presence of unpredictable amounts of contaminants, nitrogenous as well as carbohydrate. Even vigorous hydrolysis of lignin does not remove non-lignin materials. This explains the contradictory results obtained for lignin digestibility since the contamination of the lignin from faeces will be different from that of the lignin from the food, as indicated by Louw(1941) Lancaster (1943) and Forbes and Garrigus (1950b). A higher contamination of the faeces lignin fraction would produce an apparent negative digestibility, while the reverse would produce or exaggerate a positive digestibility.

(2) In the current methods involving acid prehydrolysis in the pretreatment there is some loss of absolute methoxyl due to loss of lignin.

(3) The absolute methoxyl in the lignin fraction appeared to be the least affected by the following:- (a) treatment with 72% H_2SO_4, (b) final hydrolysis of the dried lignin with dilute acid (this treatment appeared to remove methoxyl belonging to contaminants), (c) the omission of enzyme digestion and final hydrolysis in some cases (e.g. seeds hay and faeces from seeds hay), (d) the presence of associates (e.g. proteins and carbohydrates) not containing methoxyl, and associates (e.g. pectin) containing labile methoxyl, (e) evidence was obtained in support of the assumption that the recovery of lignin/

lignin methoxyl is almost complete in the acid lignin fraction provided there are no losses in filtration and final hydrolysis is not applied to the lignin residue when wet.

(4) Thus it appeared that the absolute methoxyl content of the acid lignin fraction could be taken as an index of the amount of lignin present, using a procedure similar to 'proposed method 1' to determine the absolute methoxyl of the resistant fraction of lignin, and another procedure to determine the absolute methoxyl of the total lignin. Earlier experiments suggested that the total lignin fraction could be obtained by a short-ethanol-benzene extraction followed by pepsin digestion, then 72% H_2SO_4 digestion until the cellulose reaction was negative, dilution about 6 times, filtration, washing with 0.1N HCl, then drying; methoxyl determination could be carried out directly on the dry residue or after final hydrolysis in 3% H_2SO_4 for 2 hours and washing and drying in the same manner. This procedure is designated proposed method 2. This method in combination with proposed method 1 needed to be tested in both food and faeces, to determine the necessity for low temperature drying and the effects of short cuts such as the omission of pepsin-digestion in the case of faeces.

(5) It seemed obvious that reliance on absolute methoxyl/

methoxyl content would give a more accurate measure of lignin digestibility, although it would not indicate the absolute amount of digestible lignin in a given food unless the absolute methoxyl content of pure lignin were known.

It should be noted that the methods suggested, involve a direct method of obtaining the lignin fraction, and an indirect method of determining the relative amount of lignin in the fraction. This is quite different from the earlier indirect methods of determining lignin from the absolute methoxyl content of the original plant material (Chapter 1) which are unreliable for feeding stuffs, particularly young plants, where there may be large amounts of non-lignin material containing methoxyl.

CHAPTER VI

The Preparation of Pure Lignin for Reference Purposes

Alkali lignin was found unsatisfactory as a general reference substance (Chapter IV) and the widespread belief that the methoxyl percentage in lignin increases with the age of the plant seemed to be incorrect.

The study of acid lignin (Chapter V) revealed the unreliability of figures for ash-free lignin or ash-free lignin corrected for crude protein, and it appeared that the absolute methoxyl content of a lignin fraction might be used as an index of the amount of 'pure lignin' present. Methods were formulated to determine the absolute methoxyl contents of both the lignin fraction insoluble in 72% H_2SO_4 and the total lignin; these would indicate the relative amounts of 'pure lignin' present, but for the determination of the absolute amount of 'pure lignin' present in any fraction, it is necessary to know the methoxyl content of pure lignin and for this purpose a pure reference lignin is needed.

Previous observations with ethyl aceto-acetate, (qualitative tests with phloroglucinol (p202) and alkali lignin (p.251) and limited quantitative data with proposed method 1 (p.237-9)) showed that the ester might be a more specific solvent for lignin so a study was made of the qualities of ester lignin prepared/

prepared from different feeding stuffs at different stages of growth in the hope of obtaining a pure lignin suitable for reference purposes in respect of methoxyl content. Previous preparations of ester lignin were made only from wood and on a very limited scale by Virasoro (1942, 1943).

6.1 Preliminary investigation

(i) The ester extraction procedure of Lemmel (1935) was first used, - moistening the plant material with conc. HCl (Ca.1ml/g. original material) adding the ester (5 to 10 ml/g.), stirring and filtering, and washing with the ester moistened with conc. HCl. The filtrate and washings were warmed to 45°C as this was not objectionable, and increased the yield of lignin. The extracted plant material was usually washed with alcohol to remove the remaining ester then further washed with water, alcohol and ether.

(ii) Using faeces from sheep fed on seeds hay (pretreated with ethanol-benzene and pepsin-HCl) several attempts were made to separate the materials dissolved in the ester, for example by the addition of sodium hydroxide solution (to neutralize the HCl) or organic solvents (ether, acetone, benzene, butanol, ethanol) or water, but none of these trials was a practical success. In the majority of cases two layers of solution were formed, and in some cases it was/

was possible to separate the layer containing most of the dissolved material. The dissolved material was recovered in a few cases by boiling the solution after dilution with water (in the case of NaOH solution) or by evaporation (in the case of organic solvents). Finally it was found that simply diluting the ester solution with an adequate volume of water, prevented the formation of two phases as the whole of the ester dissolved in the water phase and the dissolved material separated as fine particles. Eight volumes of water were found to be a suitable quantity and warming the water to about 45°C facilitated mixing. The precipitate was filtered off through Whatman No.50 paper, but appreciable amounts of the precipitate remained adhering to the sides of the vessels.

The light brown residue obtained after washing with water and drying gave distinct positive reactions with chlorine - Na_2SO_3 and with phenol reagent. It dissolved in cold dilute alkali, giving a dark solution similar to that of alkali lignin obtained from seeds-hay No.1 (Chap. IV) and was reprecipitated by acidifying, coagulated into flakes by warming and left a clear supernatant solution exactly as in the case of alkali lignin prepared from seeds hay 1. The material was thus related to lignin. Further purification by solution in alkali and reprecipitation gave a better physical condition and enabled the fraction/

fraction adhering to the sides of the vessel to be recovered as well as removing traces of the ester present in the first precipitate. The procedure adopted after dilution of the ester was to filter through a Gooch crucible (quicker and better than Whatman No.50 paper) wash with water, transfer the precipitate (with the asbestos mat) back into the original container, redissolve in a suitable amount of slightly warm 0.5N.NaOH, stir, filter through a Gooch and wash with portions of the alkali. A clear alkaline solution of the material was obtained, and treated in a manner similar to the final solution in the alkali lignin extraction procedure (reprecipitated and coagulated by acidifying and warming and finally filtered through a sintered silica crucible, Whatman No.50 paper disc in a Gooch crucible or small Buchner funnel).

(iii) When the extraction procedure was tested with the reagents only (conc. HCl plus ester warmed and diluted with water), a clear solution was obtained and it was concluded that no reaction could occur between the ester and the acid to form any precipitate. The transparent mixture of the conc. HCl and ester when left for 24 hours became yellowish with a tinge of brown and although Virasoro (1942) recommended 24 hours contact, it seemed undesirable to prolong the contact of plant material with the solvent in case

some/

some changes did occur.

6.2 Quality of ester-lignin from pretreated material

(i) Except in the case of immature lucerne the results tabulated in Table A36 were obtained with 2 to 5g. samples of oven dried (100-105°C) material after extraction with ethanol-benzene and in some cases digestion with acid pepsin. In treatment 1, the faeces were further extracted with conc. HCl to remove interfering substances which might otherwise dissolve in the conc. HCl used to moisten the material before adding the ester. Only a poor yield of ester lignin was obtained however, owing to the removal of a large fraction of the lignin in the conc. HCl extraction. In treatment 2, the material was very similar, but conc. HCl extraction was omitted and the yield of ester lignin was high and it contained a significantly lower nitrogen percentage and slightly higher methoxyl percentage than the Ellis acid lignin from the same material (Table A18). Comparison with the composition of the corresponding alkali lignin (Table A18) shows the ester lignin to have a lower N content but also a slightly lower methoxyl content indicating that the ester lignin was more contaminated with non-nitrogenous materials. The quality in the case of the faeces from immature pasture (treatment 3) was similar to that of the alkali lignin, but slightly

better/

better in respect of methoxyl content.

The ester extraction in treatment 2 lowered the ash-free acid lignin (as determined by a procedure similar to proposed method 2) from 31.69% of the original dry matter to 26.96% and conc. HCl extraction lowered both ester lignin yield (treatment 1 Table A36) and the acid lignin fraction (determined by Proposed Method 2, diluting the 72% H_2SO_4). These facts together with the similarities in methoxyl content and response to chlorine-Na_2SO_3 indicate the presence of identical material in these lignin fractions.

With oat straw (treatments 4 and 5a) the yield of ester lignin was high and similar to that of alkali lignin. From the residue left in treatment 5a a further yield of lignin was obtained (5b) by re-extraction with warm ester and the total yield amounted to 7.2% of the original dry matter; the absolute methoxyl in the total ester lignin amounted to 51% of that in the total acid lignin. Warming the solvent did not seem to produce ester lignin with a significantly different methoxyl percentage but the nitrogen percentage was higher. The 2nd fraction (treatment 5) was less contaminated with carbohydrates probably due to their removal in the first extraction, and the warming with the ester was possibly responsible for the increasing extraction of nitrogenous material. The quality of the ester lignin was significantly lower/

lower than that of the corresponding alkali lignin which contained 17.45% methoxyl and it seemed likely that the ester-acid mixture dissolved non-nitrogenous material which contaminated the ester lignin fraction but which was not found in the alkali lignin when the amount of nitrogen in the straw was very low (Chap.IV)

In the case of lucerne (treatments 6 and 7) ester extraction gave a very low yield of lignin with very poor quality. It did not coagulate firmly on heating the alkaline solution after acidification and the supernatant solution was not clear, although the residues were lighter in colour than those obtained by the alkali lignin extraction procedure. It was noticeable that the immature lucerne, which was not even dried but washed with water and dehydrated with alcohol, produced an ester lignin fraction of better quality than the oven-dried mature lucerne; this may have been due to the removal of interfering substances.

(ii) The results showed clearly that interfering plant constituents were present after mild pretreatment, and that the extractant was not highly specific for lignin as both nitrogenous and non-nitrogenous contaminants were present in the ester lignin fraction. The solvent mixture did not quantitatively extract lignin, and in the presence of large amounts of nitrogenous/

nitrogenous material (as in legumes) the solvent action appeared to be greatly limited; on the other hand about 50% of the lignin (as measured by absolute methoxyl) could be extracted from cereal straw containing very little nitrogen. This effect may be a physical one due to the nitrogenous material preventing the solvent from contacting the lignin, or perhaps due to the presence of a protein - lignin complex with somewhat different properties.

The figures obtained by Virasoro (1942, 1943) for the percentage of methoxyl in ester lignin from quebracho wood (previously extracted successively with ether, alcohol-benzene and in some cases with water) were 17.2% (1942) and 16.02% (1943). The latter figure is similar to that obtained for straw (Table A36) and it is likely that the ester lignin obtained by Virasoro also contained some non-nitrogenous contaminants. In order to get a high quality lignin more efficient pretreatment is necessary to remove associates soluble in the extractant or to render them insoluble.

6.3 Quality of ester lignin obtained from alkali pulp

Ester extraction of alkali pulp had not yet been attempted and it was doubted whether the ethyl aceto-acetate-HCl mixture would extract any lignin after the alkali had removed the more easily extractable lignin, particularly in the case of graminae.

The materials examined are shown in Table A37, plants designated as young being very leafy and those described as immature being cut before the flowering stage. The immature pasture, however, did contain some plants at the flowering stage and seeding stage although the crop as a whole was relatively leafy. From 10 to 90g. samples were used according to the maturity of the material and the amount of acid lignin (where known).

The materials were oven dried (100-105°C) except in the case of immature lucerne which was washed with water and dehydrated with alcohol prior to ethanol-benzene extraction and drying at low temperature. In most cases the oven dried material was extracted with ethanol-benzene before the other treatments, but with immature Italian Rye grass and Cocksfoot (treatments 10 and 12) it was found more practicable to extract the alkali pulp as the bulk was then very much less and it became possible to deal with large samples (90g. original D.M.) Alkali extraction was with 0.5N or 1.25N.NaOH. Except when otherwise indicated the/

the alkali pulp was moistened with conc. HCl (1ml/g. original D.M.) then ethyl aceto-acetate added (5-10ml/g. original D.M.) with stirring and warming by means of a water bath surrounding the container and containing initially cold water, heated up slowly. After filtering and diluting with 8 volumes of warm water the precipitate was filtered, washed with 0.1N.HCl redissolved in warm 0.5N.NaOH, reprecipitated by acidification, and filtered off after granulation. In some cases the ester-HCl extractant was used cold and moistening the dry alkali pulp with conc. HCl was avoided by mixing the acid catalyst with the ester before application. This practice was ultimately adopted to avoid contaminating the ester lignin with associates soluble in conc. HCl. The alkali pulp after extraction with ester-HCl reagent is designated 'ester pulp' and in some cases this was directly re-extracted with the ester-HCl while in others the ester pulp was subjected to another alkali extraction before a second ester extraction.

(i) Mature plants (treatments 1 and 2, Table A37) With oat straw the ester lignin quality was better than when alkali extraction was excluded (Table A36); the nitrogen content was almost negligible, and the methoxyl percentage was even higher than that of alkali lignin (17.45%) prepared from the straw (Table A114, Tr. 1) although the methoxyl percentages in the corrected/

corrected lignins were practically identical (18.10% and 17.89%). Thus the ester lignin was purer than the alkali lignin, and a better reference lignin. Since the crude protein correction produced closer agreement between the percentages of methoxyl in alkali and ester lignins, it seemed that the correction was justifiable, and ought to be applied. The ester lignin had the advantge of being more easily prepared than alkali lignin, and the use of alcohols was not involved. The percentage of methoxyl in the ester lignin from straw was probably higher than any figure recorded for straw lignin or alkali lignin from woods, or ester lignin prepared from hard woods by Virasoro (1942, 1943). The yield of ester lignin was lower than when alkali extraction was excluded, because of the removal of much of the extractable lignin during the alkali treatment (40% of the methoxyl present in the acid lignin was removed by the alkali extraction). When related to the amount of lignin remaining in the alkali pulp the percentage extraction by the ester would be very much greater.

With the mature lucerne the results differed markedly from those obtained with alkali lignin or with ester lignin obtained without alkali pretreatment; whereas those lignins were of very poor quality, the ester lignin from the pulp was comparable in quality with the ester lignin from oat straw pulp containing/

containing a somewhat higher methoxyl percentage. The ester lignins obtained from straw and lucerne in treatments 1 and 2 resembled one another; they were of creamy colour, and coagulated firmly when their solutions in alkali were acidified and heated, leaving transparent supernatant solutions. There was no support for Bondi and Meyer's claim that legumes contain lignins with only 5% methoxyl. It is interesting to note that the alkali lignin prepared from mature lucerne (Chap. IV) contained 4.24% methoxyl, and its yield contributed only about 6% of the methoxyl in acid lignin, whereas ester lignin from the alkali pulp contained 18.14% methoxyl amounting to 20.9% of the methoxyl in acid lignin. It is obvious that the poor quality of alkali lignin was due to contamination with both nitrogenous and non-nitrogenous material; the alkali lignin contained 6.97% N but even after correction for crude protein the methoxyl percentage only rose to 7.51% in the corrected lignin (Table 14, Tr. 8). Since the ester lignin contained 18.14% methoxyl and only 0.41% nitrogen practically all the nitrogen in lignin may be regarded as a contaminant as was indicated by previous results (p260-1 and 327).

There was a marked difference between the behaviour of cereal lignin and legumes lignin towards extraction/

extraction with alkali and aceto-acetic ester; in the former case the yield of alkali lignin was much higher than the subsequent yield of ester lignin (as indicated by methoxyl yields relative to the total methoxyl in the acid lignin) but the reverse was true in the latter case. This appeared to be due to the low solubility of legume lignins in alkali, as indicated by the results obtained with young clover and seeds hay (Chap. IV). Also in the case of lucerne the alkali extraction appeared to remove a high proportion of associates, particularly proteins, leaving a pulp suitable for the extraction of lignin in a much purer form. The success of the ester-HCl mixture was due to its ability to extract a lignin fraction (resistant to relatively mild alkali treatment) after the removal of soluble interfering associates. This solvent appeared to be more selective than the alkaline solution which failed to produce a high quality lignin even after repeated extractions (Chap. IV)

It was concluded that ester-lignin could be obtained from mature legumes and cereals in a relatively pure state and showing similar properties; this could possibly be used as a general 'reference lignin'. The methoxyl percentage was higher than recorded figures for alkali lignin from woods and approached/

approached the methoxyl percentage recorded recently for 'native lignin' from hard woods.

(ii) Faeces from sheep fed on timothy hay. Trials with faecal material were carried out, to study the nature of the ester lignin, and to see if any demethoxylation took place during passage through the animal. It was realised also that faecal material would contain a much higher lignin percentage than the food and this would improve the yield of ester lignin.

In treatment 3 (Table A37) the alkali extractives obtained from the faeces of sheep fed on Timothy hay were retained for the preparation of alkali lignin. The ester lignin from the pulp was of similar quality to the ester lignin obtain from mature timothy (Table A38) although the nitrogen percentage was somewhat higher and the methoxyl percentage slightly (but not significantly) lower. It was therefore unlikely that any demethoxylation had occurred in the digestive tract and the recorded decrease (Bondi and Meyer) in the methoxyl percentage of acid lignin during digestion was probably due to greater contamination of the faeces lignin. As the lignin from timothy straw (containing 0.42%N, Table A38) was practically identical with that from faeces derived from timothy-hay containing 1.18% N (treatment 3 Table A37) it appears that the maturity of the plant/

plant does not affect the methoxyl percentage in the lignin. The slight difference between the two lignins was probably due to the presence of some impurities in the faeces lignin extracted by the conc. HCl used to moisten the alkali pulp. The experiment was repeated (treatment 4a) using cold ester-HCl extraction and the pulp remaining was then subjected to warm extraction (treatment 4b); the ester lignins from treatments 4a and b were of identical quality, indicating that warm extraction was not objectionable, but the methoxyl percentage was noticeably lower than in treatment 3, indicating that there may be a variable fraction of impurities extractable by the acid or ester-acid mixture and precipitated with the ester lignin, causing some variation in the methoxyl percentage. The ester lignin was found to be practically ashless; this was checked later with other plants and did not exceed 1%.

The alkali lignin prepared from these faeces was contaminated (1.65% N and 14.65% methoxyl in corrected lignin (Chap. IV) and obviously unsuitable for reference purposes.

(iii) Faeces from sheep fed on immature pasture

In treatments 5a, 6a and 6b (Table A37) the alkali extraction used was milder than in treatments 3 and 4 with faeces from timothy hay, as it was expected that/

that alkali extraction would considerably reduce the lignin content of the alkali pulp. In treatments 5a and 5b the ester lignin was filtered off directly without final purification in alkali and the yield was higher than in treatments 6a and 6b. The ester lignin from treatment 5a appeared to be of low quality as the nitrogen percentage was high, but the second yield of ester lignin from the pulp (treatment 5b) obtained with warm solvent, was lower in nitrogen content, and although the methoxyl percentage in the corrected lignin (13.47) was lower than that of ester lignin from mature plants, it was much higher than that of alkali lignin (9.42) or acid lignin (9.89) prepared from the alkali pulp (Chap. IV); The ester pulp from treatment 5b was used to attempt a third extraction of ester lignin (treatment 5c) but only traces were obtained. The ester lignin from treatments 6a and 6b was of poorer quality than that from 5b and it is probable that the first ester extracts in 6a and 6b contain appreciable amounts of contaminants whilst the later extracts from ester pulp (as in 5b) contain much less. The contaminants of ester lignin appear to be mainly non-nitrogenous as the following ~~lignin~~ figures show.

N %/

	N% in Lignin residue	Methoxyl % in lignin residue	Methoxyl % in corrected lignin.
Alkali lignin	3.50	7.36	9.42
Ester lignin	1.21	8.32	9.00

The ester pulps from treatments 6a and 6b were combined and extracted with boiling 1.25N.NaOH prior to further extraction with ester (treatment 6c). This treatment gave a much less contaminated lignin than treatment 5b, and the percentage of methoxyl in the corrected lignin was of the same order as that in 'native lignin' from soft woods. The results also indicated that the higher contamination of the first yields (Treatment 6a and 6b) was due to the inadequacy of the milder alkali extraction, for removing contaminants. The more concentrated NaOH used in treatment 6c appeared to remove associates which may have been linked with the lignin or physically surrounding it, preventing extraction by the ester. On the basis of methoxyl content the yield from treatment 6c was greater than that extracted in 6a or 6b.

In the last experiment with faeces from immature pasture (Treatments 7a,b,c), 1.25 N.NaOH was used for 1 hour in an attempt to shorten the alkali extraction procedure. In treatment 7a, the alkali pulp was filtered off and washed with hot water only (not hot alkali)/

alkali), and the final ester lignin was precipitated in the usual manner with only one purification by reprecipitation from alkali. In treatment 7b, however, the alkali pulp was washed in the usual manner with the hot alkali, and the precipitated ester lignin was dissolved in alkali and carried through the procedure used for alkali lignin extraction (Appendix p. 8). The yield from treatment 7a was not high, and as the quality was similar to 6a, it seemed that repeated extraction with alkali was better than one short extraction. In treatment 7b a marked improvement in quality was obtained but the yield was greatly reduced owing to the several additional operations. There was an effective reduction of non-nitrogenous contaminants but the procedure was tedious.

In treatment 7c a double alkali extraction was made and the ester was mixed with the conc. HCl before addition to the dry alkali pulp: both the yield and the quality of the lignin were better than in treatment 7b, and the percentage of methoxyl in the corrected lignin was similar to that obtained with faeces from Timothy hay in treatments 4a and 4b, although the nitrogen content was higher.

The results with immature pasture showed clearly that a low percentage of methoxyl in a lignin preparation was due to the presence of contaminants. It was possible/

possible, by several purifications, to prepare a series of ester lignins with methoxyl percentage varying from 9.00 to 16.85%.

The purest preparation (16.85% methoxyl) contained 0.73% nitrogen approaching the values found for mature plants and the methoxyl percentage was similar to the figures recorded for lignin preparations from hard woods. It appears safe to conclude that the pure lignin in mature plants is identical with that in immature ones and there is probably no change at any stage of growth.

(iv) Immature lucerne

The results with immature lucerne (treatment 8, Table A37) confirmed the final conclusions from the previous experiment. There was no difficulty in preparing a high quality ester lignin from this material which contained 3.67% N and was at an earlier stage of growth than the pasture grass fed to obtain the faeces used in treatments 5 to 7. The reason for this was the difference in the behaviour of legumes and gramines towards alkali extraction. The pasture grass contained 63% of grasses and 37% of legumes (on a dry weight basis) and alkali extraction of the faeces would greatly reduce the lignin content of the alkali pulp; the yield of ester lignin extracted from the pulp would, therefore, be low and the amounts of contaminants would be relatively higher. With the/

the immature lucerne however relatively smaller amounts of the lignin would be removed by alkali extraction, the ester lignin yield would be higher and in consequence the amounts of contaminants would be relatively lower. This is clearly seen by comparing treatments 6a and 8 which were similar, although one hour boiling with 0.5N.NaOH (in the case of lucerne) was perhaps more drastic than long extraction at 85°C with the same reagent (treatment 6a). The recovery of methoxyl in the lignin from immature lucerne (treatment 8) was 17.7% of the methoxyl contained in the total acid lignin whilst the recovery in the case of faeces from pasture (6a) was only 3.3%; the latter figure would remain the same if the results were based on the original food, since the biological treatment of digestion may be regarded as a pretreatment. The absolute methoxyl in the total acid lignin from the pasture (0.658%) was very similar to that for the lucerne (0.687% of original D.M.) and thus the absolute amount of ester lignin extractable from lucerne would be about 5 times as great as that extractable from the same quantity of pasture dry matter.

(v) Immature grasses.

Both the Italian ryegrass (treatments 9 and 10, Table A37) and the Cocksfoot (treatments 11 and 12) were

second cuts obtained at the end of October and represented about 4 months growth, in a dry season; they were not in flower and the cocksfoot was growing vigorously. The Italian ryegrass contained 1.26% N (in the D.M.) and the Cocksfoot 1.77%.

Treatments 9 and 11 were the same as used for immature lucerne, but the alkali pulp was extracted with conc. HCl before the addition of the conc. HCl-ester mixture. The yields were low and the lignin quality was similar to that from pasture faeces in treatment 6a, the Italian ryegrass lignin being rather purer than that from Cocksfoot. In treatments 10a and 12a, (large samples ~90g.) a combination of enzymatic and mild alkali treatments was employed and the ester and HCl were mixed before addition to the dry pulp. The improvement in the quality of the ester lignin obtained was noticeable; although the contamination with non-nitrogenous materials was reduced, the nitrogen content was still appreciable in the case of Cocksfoot.

In treatments 10b and 12b, the ester pulps from 10a and 12a were re-extracted with the mixture of ester and conc. HCl and small yields were obtained of a substance which did not resemble ester lignin. Its coagulation during final precipitation was not firm, its colour was dark and the wet filtrate appeared/

appeared to be relatively bulky but diminished greatly in size on drying indicating physical properties different from those of good quality ester-or alkali-lignin. The methoxyl precentage in the product was very low indicating that it was probably a mixture containing a small fraction of lignin and a large fraction of methoxyl-free contaminant. This was the first direct evidence that contamination could occur in ester lignin preparation.

(vi) Young plants

In young oats and clover (treatments 13, 14 and 16 Table A37) the ester lignin contained about 10% methoxyl, but in treatment 14 when a large sample of young oats was treated with stronger alkali the yield was very low, although the methoxyl percentage in the lignin residue increased.

With young timothy (the plant growth was short but had become coarse) the alkali pulp was extracted with conc. HCl before the treatment with ester (treatment 15) and the methoxyl percentage was higher than in the other cases but the yield was low. The methoxyl percentages in the corrected lignins from treatments 13 to 15 would be expected to be higher than the uncorrected lignin figures shown in Table A37. The results indicated that the percentage of methoxyl in ester lignin from young plants extracted into the range obtained in immature plants, and even higher figures were obtained/

obtained in some cases. This showed that the variations were due to the presence of contaminants rather than to any actual difference in the methoxyl percentage in the lignin of young and older plants. The combined results lead to the important conclusion that the percentage of methoxyl in lignin is most probably constant throughout the growth of the plant, and any apparent decrease in methoxyl percentage (provided the conditions of extraction do not produce demethoxylation) is due to contamination. The ester lignin from mature plants appeared to be the best 'pure lignin' for reference purposes.

6.4 Ester lignin for reference purposes.

Mature plants were used to obtain ester lignin for reference purposes and particular attention was paid to possible differences between plant species, particularly grasses and legumes.

6.4 (i) Procedure for ester lignin preparation.

(a) Ethanol-benzene extraction :

Finely milled, dried plant material (30 - 50g.) was extracted with ethanol-benzene (1 vol. 95% alcohol : 2 vols. benzene) for 6 hours in a Soxhlet apparatus, (syphoning 6 times per hour) then washed in the extraction thimbles with alcohol and ether, using gentle suction (the thimbles were fitted directly to filter flasks and suction applied). The samples were then dried in a non-sparking electric oven, either in the thimbles or after transfer to paper scoops (for quick drying).

(b) Alkali Extraction:

After transfer to a flat bottomed flask of suitable size, hot 0.5N NaOH was added (20 ml. per g. dry material) in small portions to facilitate uniform distribution of the plant material, the solutions then brought to the boil and kept boiling gently for 40 minutes in the case of grasses, and 60 minutes for legumes. The shorter period of boiling with grasses was to reduce the losses in

lignin./

lignin. Incubation overnight at 85°C could be used as an alternative with grasses but boiling was preferred for legumes as it removed proteins more efficiently. After alkali treatment the plant material was filtered through cheese cloth, washed several times with the hot alkali solution until the washings were only slightly coloured, then washed successively with hot water, 0.1N HCl, water, alcohol and ether, and finally dried. In some cases the plant residue after alkali treatment required remilling to break down the lumps, but it was later found that stirring the plant particles in the organic solvent during washing with alcohol and ether produced a desirable physical condition and made remilling unnecessary.

(c) Extraction with ethyl aceto-acetate.

A freshly prepared solution of ethyl aceto acetate (5 vols.) and conc. HCl (1 vol.) was added at the rate of 5 volumes per g. of original material to the alkali pulp in a beaker and placed in cold water bath. It was gently heated to 45°C with stirring, then left to cool (to prevent loss of HCl fumes during filtration), and filtered by suction through Whatman No. 50 paper in a Buchner funnel, washing twice with suitable volumes of the reagent. The filtrate was diluted with 8 volumes of warm water,

stirring/

stirring quickly to dissolve the ester in the water and prevent the formation of an emulsion. The precipitated lignin was left to settle, then filtered off through an asbestos mat in a Gooch crucible or small Buchner funnel, washed with 0.1N HCl and re-dissolved in a suitable volume of warm 0.5N - NaoH (together with the asbestos and in the original container to avoid loss of any ester lignin sticking to the sides); the alkaline solution was filtered, the lignin reprecipitated by the addition of 0.5N HCl and then the precipitate granulated by heating in a water bath gradually brought nearly to boiling point. The ester lignin was finally filtered off, washed with water to remove HCl, and dried.

6.4 (ii) Ester lignin from mature plants.
(Table A38).

(a) Soft Woods.

The lignin of soft woods has been extensively studied and is considered to be of lower methoxyl content than that of hard woods owing to the absence of syringyl radicals from the molecules. The 'native lignin' of soft woods was repeatedly studied by Brauns and co-workers (Chapter I. P 22) but no preparation of ester lignin from soft woods appears to have been made.

The ester lignin prepared from Scots pine

(Treatment/

(Treatment 7, Table A38) contained 15.35% methoxyl, which was very similar to the figures recorded by Schubert and Nord (1950a) for native lignin from white Scots pine (15.3%) and within the range (14.7 to 15.5%) recorded by Brauns (1940) and Brauns and Hibbert (1935) for native lignins from soft woods. This result supported the use of ester lignin as a reference, and possibly the ester-HCl extraction procedure was mild and did not affect the lignin quality.

(b) Gramines and Legumes.

The range of methoxyl percentage in the corrected lignin (reference lignin) from four different species of gramine was very narrow (from 17.72% to 18.13%) and it seems probable that the lignins of all gramines are of identical methoxyl content. The nitrogen percentage was less than 0.2% in three cases, and 0.34% in the other (Cocksfoot), probably because this last sample was not very mature; it contained a relatively high nitrogen content (0.95%), and the alkali extraction was mild.

The methoxyl percentages in the corrected lignins from two legumes of different species were practically the same, and slightly higher than the percentages for gramines. The nitrogen contents of the ester lignins were about twice as great as

in/

in the case of gramines, but did not exceed 0.60%.

The average methoxyl percentage in the corrected lignin from the four gramines was 17.94 ± 0.21 and the corresponding value for the two legumes was 18.59 ± 0.02, the differences being significant at the 5% but not at the 1% level. It may be that legume lignin naturally contains a slightly higher methoxyl percentage than the lignin in grasses and cereals. The recovery of ester lignin (as indicated by the methoxyl recovery relative to the methoxyl in the acid lignin) from the legumes was about twice as great as the recovery from the gramines and, being in nearly all cases between 10 and 20%, this might well be representative of the lignin present. The percentage recovery of ester lignin based on the amount of lignin present in the alkali pulp may be considerably greater, particularly in the case of the gramines. Other lignins like 'native lignin' frequently form only a very small fraction (about 8%) of the total lignin and so may be unrepresentative.

The methoxyl percentages obtained were higher than any recorded for grasses, cereals and legumes and approached the figure (19.5) recorded for native lignin from hard woods (Buchanan et al, 1949). It may be that the methoxyl percentage in the lignin of hard/

hard woods, cereals and legumes varies only within narrow limits.

6.4 (iii) 'True lignin' and 'Methoxyl Conversion Factor'.

The results indicated the constant nature of the methoxyl percentage in lignin and the ester lignin prepared from mature gramines and legumes very probably represented the purest lignin ever obtained from these plants. Previous studies with acid lignin (Chapters II to V) indicated the unreliability of acid lignin figures expressed on the basis of either ash-free lignin or corrected lignin, and the greater reliability of the absolute methoxyl present in the acid lignin fraction; there was, however, no reference lignin to relate absolute methoxyl content to true lignin content. It is now suggested that ester lignin may be used as the reference for this purpose and it is preferable to use for each plant species an ester lignin prepared from mature plants of that species until more extensive data is available to give a more accurate picture of the range of methoxyl percentages in the lignin from different species. One average figure may be satisfactory for all agricultural plants, or perhaps one for gramines and one for legumes may be more accurate.

The/

The methoxyl conversion factors for 'true' lignin given in Table A38 were calculated from the methoxyl percentages in the reference lignins (ester lignins corrected for crude protein) and the use of these factors (or any suitable average) in conjunction with data for the absolute methoxyl present in the acid lignin fraction would enable the 'true' lignin content to be determined.

CHAPTER VII

Final Procedures for Lignin Determination

In Chapter V, it was suggested that the absolute methoxyl in the acid lignin fraction might be a more reliable index of the amount of 'pure' lignin present, and in Chapter VI, it was shown that the ester lignin prepared from mature plants was relatively 'pure' lignin and could be used as a reference lignin. It seemed possible, therefore, to obtain a fairly accurate measure of the amount of "true lignin" by multiplying the absolute methoxyl present in the acid lignin fraction by a conversion factor obtained from the percentage of methoxyl present in the 'reference lignin' from the plant.

Two methods for acid lignin were proposed, giving in one case the fraction insoluble in 72% H_2SO_4 (proposed method 1) and in the other the total lignin (proposed method 2). A further study was made of these methods in order to evolve a more rational procedure and some aspects of the determination of the methoxyl group were also examined.

7.1 Accuracy of Methoxyl Determination :-

In general the presence of a high nitrogen content in prepared lignin was associated with a low methoxyl percentage which persisted even after

correction/

correction for crude protein; this was attributed to the presence of non-nitrogenous contaminants.

It was also suspected that the presence of nitrogenous material in the lignin fraction might have a reducing effect on methoxyl during the reactions of methoxyl determination. This was shown to be unlikely as the addition of pure protein to plant material during contact (with 72% H_2SO_4) in acid lignin determination, greatly increased the nitrogen content but did not reduce the absolute methoxyl. (Chapter V. P326). A further test was made adding pure protein to an equal amount of vanillin before methoxyl determination and the recovery of methoxyl was satisfactory.

Phillips and Goss (1935) raised the question as to whether a methyl group attached directly to carbon could be split by hydriodic acid in the methoxyl determination. This possibility was tested with a methyl pentose (shamnose) which gave the negligible value of 0.096% methoxyl, indicating that such methyl groups would not interfere with methoxyl determination.

7.2 Factors affecting acid lignin preparation.

(a) The effect of drying temperature, on the insoluble factors of acid lignin prepared by proposed method 1 was tested with a faeces sample

from/

from sheep fed on immature pasture grass. The results (Treatment 1, Table A39) indicated no practical difference between high temperature drying (105°C) and drying at 50°C after alcohol dehydration in respect of lignin yield, quality and absolute methoxyl content.

(b) The effect of final hydrolysis with 3% H_2SO_4 on the absolute methoxyl in the total acid lignin was examined in treatments 2, 3, 4 and 5 using an alkali pulp from seeds-hay 2 (Chapter IV) and samples from a sheep digestibility trial with timothy hay. In the case of the alkali pulp the whole lignin (filtered in a Gooch) was used for methoxyl determination, but with the other samples the dry lignin residues were subdivided, and portions subjected to final hydrolysis. Filtration of the lignin fractions before hydrolysis was rather slow when sintered glass crucibles were used.

The difference (about 3%) between treatments 2a and 2b was not significant, indicating that the final hydrolysis had no effect on the absolute methoxyl present in the lignin fraction from the alkali pulp of seeds-hay.

With the timothy hay digestibility samples, the recovery of absolute methoxyl after final hydrolysis (Treatments/

(Treatments 3b, 4b and 5b) was 95.7%, 104.1% and 98.5% from food, food residue and faeces lignins respectively, and the lignin quality was significantly improved, with the fall in nitrogen content producing a noticeable reduction in ash-free lignin.

These two experiments confirmed the indications of earlier experiments (Chapter V. P312), showing clearly that the absolute methoxyl in the total lignin is not affected by final hydrolysis provided methoxyl-containing contaminants are removed by a pretreatment preferably including pepsin-digestion. It is also concluded, however, that final hydrolysis is unnecessary when procedures such as methods 1 and 2 are based on the absolute methoxyl in the acid lignin fractions.

7.3 Comparison of yields and qualities of soluble and insoluble lignin fractions.

The digestibility trial samples (Treatments 3, 4 and 5, Table A39) were used for this purpose, the figures for the soluble lignin fractions being obtained by difference. The soluble lignins were of better quality (as indicated by nitrogen and methoxyl percentages) than the insoluble lignins, and the total lignins were, of course, intermediate. The insoluble lignins in food, food residues and faeces were of similar/

similar quality but the corresponding soluble lignins showed rather more variation and, as the figures were obtained by difference, greater variation was to be expected.

With the food and foodresidue the soluble lignin fraction (ash-free or corrected) was usually less than 45% of the total lignin, but on the basis of absolute methoxyl content it formed more than 50% of the total, due to the soluble fraction being richer in methoxyl. In the faeces the soluble fraction of the lignin formed 31% of the total on an ash-free basis, 87% of the total in terms of corrected lignin and 43% of the total on the basis of absolute methoxyl content. The proportion of soluble lignin in the total lignin was thus lower than in the lignin of food and food residues, suggesting that some changes occurred in the digestive tract reducing the proportion of the soluble fraction.

The fact that the total lignin was a mixture of at least two fractions of different qualities was, in itself, evidence that the acid lignin was an impure product, and that the current methods relying on the weight of this product are unreliable. The better quality and high proportion of the soluble lignin made it important that this fraction should be included in any quantitative determination of lignin

as/

as indicated in Chapter V. The resemblance in quality between the acid lignin fractions of food and faeces indicated that no demethoxylation of lignin occurred during passage through the digestive tract in confirmation of the belief that rupture of the ether linkage would not take place very easily.

7.4 Combined method for the preparation of both soluble and insoluble fractions of acid lignin.

When it is desired to study the digestibilities of the soluble and insoluble lignin fractions proposed methods 1 and 2, without final hydrolysis, would give the total lignin and the insoluble lignin but the soluble fraction would not be determined directly. Moreover, the necessity for two separate complete analyses would involve a considerable amount of work. Attempts were, therefore, made to combine the two procedures by making it possible to filter the 72% H_2SO_4 solution in the proposed method 2 without dilution; the filtrate would contain the whole soluble lignin fraction which could be precipitated by dilution, filtered, washed with 0.1N HCl and dried before continuing as in proposed method 2 after dilution of the 72% H_2SO_4; the fraction insoluble in 72% H_2SO_4 could be washed with the acid, then with H2O and dried before continuing

as/

as in the proposed method 1.

It was noted that the addition of drops of conc. HCl to 72% H_2SO_4 helped to break down the lumps formed when pure proteins were treated with 72% H_2SO_4 (Chapter V. Sect.7,p.323), and it was thought that the presence of some HCl might improve the physical conditions of the plant material in 72% H_2SO_4 and facilitate filtration. This proved to be the case; by the addition of a few drops of conc. HCl (2-3 drops per 30 ml. H_2SO_4 usually used for 1g. original sample) a few minutes before filtering the 72% H_2SO_4, then stirring, it was possible to filter through asbestos in a Gooch crucible (without previous extraction of the plant material with conc. HCl) although filtration was a little slower than in proposed method 1 (where the plant material is extracted with conc. HCl before treatment with 72% H_2SO_4). Before adopting this procedure the effect of the addition of drops of conc. HCl to the 72% H_2SO_4 on the quality of the lignin was examined with the results indicated in the table overleaf.

Although the addition of conc. HCl may reduce nitrogenous contamination, improving the lignin quality, it did not appear to affect the absolute methoxyl content.

The combined procedure, designated proposed

method/

	% N in Ash-free Lignin	% Methoxyl in Corrected Lignin	Absolute Methoxyl
A. Modified Ellis method	1.84	12.1	0.890
B. As A + drops conc. HCl added to 72% H_2SO_4	1.33	13.9	1.004
C. Proposed method 2 + final hydrolysis (wet residue)	1.22	13.9	1.40
D. As C + drops conc. HCl to 72% H_2SO_4	1.00	13.01	1.26

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Method 3, was tested with timothy hay and faeces in Treatments 3e and 6 of Table A39. The soluble and insoluble fractions obtained from the hay in treatment 3e were of practically the same qualities as the corresponding fractions obtained by the separate procedures (Treatments 3a, c and d); the similarity was particularly noticeable with the absolute methoxyl figures as indicated below:-

	Soluble	Insoluble	Total
A. Lignin fractions obtained by methods 1 and 2	0.825	0.813	1.638
B. Lignin fractions obtained by combined method	0.823	0.788	1.611
Percentage difference $(\frac{A - B}{A} \times 100)$	0.24	3.0	1.6

In treatment 6, using a faeces sample derived from timothy hay, filtering of the strong acid was quicker than in the case of the hay sample. The amounts and quality of the different lignin fractions from this sample were similar to those obtained from the other faeces sample by the two methods 1 and 2. (Treatments 5a, 5c and 5d).

It was also observed that the humin precipitate of the soluble lignin fraction obtained after dilution of the 72% H_2SO_4 settled down after a few hours leaving a clear supernatant solution which could be filtered very rapidly.

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For routine analysis it was desirable to speed up the filtration of the 72% H_2SO_4 solution in the combined method. Trials were made with glass wool, and it was found that when cut with scissors into small fragments of a few millimeters in length, the material would form a satisfactory filtration medium in a Gooch crucible. With practice it was easy to judge the amount required to produce a layer of about 3 - 4 mm. thickness, and this was placed in the crucible; some of the strong acid was then added and very gentle suction applied, finally pressing the glass wool with a glass rod to spread it evenly and keep it firmly attached to the walls of the Gooch crucible. This filter medium was very successful and, unlike asbestos, did not become over compact and slow filtering if the crucible was allowed to become empty of liquid during the filtration process.

It was also found possible to ignite the glass wool mat with the soluble lignin fraction at 600°C (the temperature usually used for ashing lignin) to obtain the ash-free lignin without melting the mat or spoiling the determination. A test was made of the effect of heat on glass wool, washed and dried at 100° - 105°C, then subjected to 650°C in a muffle furnace for 6 hours. The original dry weight of the glass wool was 0.314g. and the final weight 0.313g.

indicating/

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indicating a negligible (0.3%) loss on ignition.

7.5 Final Tests with proposed method 3.

7.5 (i) Effect of temperature of drying immature and young plants.

Young timothy (one month old, 15 cm. in length) immature pasture (37% clover) and sheep faeces derived from the immature pasture were examined (Treatments 1, 2, 3, Table A40) after drying at 105°C and also after alcohol dehydration and drying at 50°C. The 'true' lignin present in the acid lignin fraction in each case was calculated from the absolute methoxyl content using an appropriate methoxyl conversion factor obtained from the percentage of methoxyl in the ester lignin (Table A38). The factor for immature pasture (containing 37% legumes) was calculated from the average methoxyl percentage in the ester lignin of two gramines and the average for two legumes, due to weight being given to the greater proportion of gramines in the mixture.

The results showed no practical differences to justify the low temperature drying. Slight differences occurred in some cases but they were within normal variation for duplicate analyses; this was also apparent from the fact that the figures for absolute methoxyl or 'true' lignin for samples dried at 105°C were in some cases slightly less than the

figures/

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figures obtained for low temperature drying; these differences were, of course, not significant.

It was also noted that the acid lignin fractions from immature pasture were similar in quality to the corresponding fractions from the faeces (Treatments 2 and 3, Table A40), and the soluble lignin was of better quality than the insoluble, particularly in respect of methoxyl percentage. The nitrogen content of the soluble fraction was, however, relatively high, and not a great deal less than in the insoluble fraction, probably due to the high nitrogen content of the original material compared with Timothy hay (Table A39) and to the physical conditions of the immature grass being more favourable for the solution of protein material in the 72% H_2SO_4. The insoluble lignin was obviously more contaminated with non-nitrogenous materials. The soluble lignin in the food formed about 50% of the total lignin (measured as absolute methoxyl) but in the faeces it formed only about 30% of the total, indicating that some changes had occurred in the digestive tract to diminish the proportion of the soluble fraction.

2.5. (ii) Effect of omitting pepsin digestion with faecal material and mature plants.

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The effect of omitting pepsin digestion in the analysis of faeces was examined in treatments 3b and 3c (Table A40) and the effect with oat straw in treatments 4a and 4b. The results showed no practical effect on the absolute methoxyl obtained. The greatest difference was between the absolute methoxyl in the soluble fractions from oat straw, (1.11 ± 0.021 and 1.19 ± 0.038) and this was not significant (P more than 0.10). It was concluded that the omission of pepsin digestion provided a good short cut when dealing with faecal material or mature straws, but was not advisable in other cases.

It should be noted that in the case of straw the qualities of the soluble and insoluble fractions of acid lingin were very similar; the soluble fraction contained a lower nitrogen percentage but the methoxyl percentages were practically identical. The soluble fraction formed rather more than 50% of the total lignin.

7.6 Final Procedure for Determination of Lignin.

7.6 (i) Outline of the Method.

1. Acid lignin fractions are prepared from dried samples as required :-

(a) If only the total lignin is required it is obtained by proposed method 2 without final dilute acid hydrolysis.

(b)/

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(b) If the lignin fractions, soluble and insoluble in 72% H_2SO_4 are to be separately determined they are obtained by proposed method 3, and in this case the total lignin is obtained by summation.

2. Pepsin digestion in the case of faeces samples, or mature straws is omitted from proposed methods 2 and 3.

3. The methoxyl in the acid lignin fraction is determined in the whole residue (filtered in a Gooch crucible) and expressed as a percentage of the original dry material.

4. The methoxyl conversion factor is preferably obtained from the percentage of methoxyl in the reference lignin ~~and~~ prepared from the mature plant by ester extraction as outlined on p.355. The average conversion factor for 4 gramines was 5.57 and that for two legumes was 5.38. For mixed foods and grasses an average factor of 5.5 may be used until more knowledge is available regarding the range of correction factors for different plant species.

7.6 (ii) Detailed description of the procedure.

1. Reagents : 72% H_2SO_4 (W/W), 0.1N HCl, 1% pepsin (B.D.H.) in 0.1N HCl, ethanol-benzene mixture (1 vol. 95% alcohol : 2 vols. benzene).

2. Procedure.

(a)/

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(a) Ethanol-benzene extraction

Plant material, oven dried and finely milled (1 - 2g. of food or ½ - 1g of faeces) is extracted with ethanol benzene for 6 hours in a Soxhlet apparatus, syphoning 6 times per hour. With the aid of suction the samples are washed twice with small portions of 95% alcohol, followed by two small portions of ether, and dried at 45°C.

(b) Pepsin digestion.

The residue from (a) is transferred to a 100 - 250ml Erlenmeyer flask, 40 - 80ml. pepsin HCl solution added, and the flask stoppered and incubated overnight at 40°C. The residue is then filtered through a Whatman No. 50 paper in an ordinary funnel fitted to a filtering flask, and using gentle suction it is washed with distilled water, alcohol and ether, then dried.

(c) Treatment with 72% H_2SO_4

The residue from (b) is transferred quantitatively to a 50 - 100ml. beaker, and 72% H_2SO_4 (30ml. per 1g. original material) added in small portions, mixing with a glass rod. The mixture is kept at room temperature, (not above 20°C) with occasional stirring for about three hours until a spot test on a small drop of the 72% H_2SO_4 mixture gives no blue colour with/

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with Iodine solution. Two drops of conc. HCl are added, and the mixture stirred gently.

(d) Final treatment.

For the determination of total lignin the mixture is diluted with Ca 6 volumes of water, filtered through asbestos in a Gooch crucible (3 - 5 cm. bottom diameter and 4 cm. tall), washed with 0.1N HCl until free from H_2SO_4, and dried at about 40°C.

ALTERNATIVELY, for the separate determination of the lignin fractions soluble and insoluble in 72% H_2SO_4 the mixture is filtered without dilution through glass wool in a Gooch crucible. (The glass wool is cut into small pieces of a few mm. in length). The insoluble residue is washed with small portions of the strong acid until the filtrate is clear, then washed with water and dried (insoluble fraction). The strong acid filtrate is diluted with Ca 6 volumes of water, left to settle, then filtered through asbestos in a Gooch, washed with 0.1N HCl, and dried as above at Ca 40°C. (soluble fraction). The dry residues are used for methoxyl determination (Appendix P 5).

(e) Calculation of 'true lignin'.

This is obtained by multiplying the absolute methoxyl present in the lignin fraction (expressed as

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a percentage of the original material) by the appropriate methoxyl conversion factor.

Remarks :

1. When it was desired to study the qualities of the lignin fractions, determinations of ash-free lignin and nitrogen were also made (Chapter 2) with separate samples using the whole lignin residue in each case. The former required that the dry residues (after being washed with 0.1N HCl in the cases of total lignin and soluble lignin) should be rewashed with distilled water until free from HCl before drying at 100 - 105°C, weighing and igniting at 600°C to obtain the ash-free lignin by loss in weight.

2. Where multiple determinations were required with a single sample, a large quantity (10 - 20g.) was pretreated, and a fraction equivalent to $\frac{1}{2}$ to 2g. of the original material taken for reaction with 72% H_2SO_4 and subsequent treatments.

7.7 Suggested Scheme for the Analysis of Feeding Stuffs.

It is suggested that the fractions, 'crude fibre' and 'N-free' extractives used in routine analysis might be better replaced by 'lignin' and 'crude carbohydrate' fractions, the former being determined directly, and the latter obtained by difference after the summation of crude fat, crude protein lignin and ash. Whether

or/

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or not it would be necessary to determine cellulose in the 'crude carbohydrate' fraction is uncertain. The system suggested would be superior from a chemical point of view to the division of lignin between the 'crude fibre' and N-free extractives. Biologically it would also be superior if lignin proved to be indigestible or the least digestible fraction of the food.

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CHAPTER VIII

The lignin content of some forage plants

8.1 Preparation of plant materials.

Most of the samples examined (Table A41) were obtained from special plots sown down at Boghall Experimental Farm early in May, 1950; the Italian ryegrass and cocksfoot, (after being cut on 30th June) failed to reach the flowering stage so very mature samples (straw) were obtained from other sources and the sample of timothy in full flower (hay) was grown in an earlier year in connection with a different experiment. In addition to the species grown in the special plots, samples of lucerne, oats, mixed pasture herbage and pine sawdust were also examined, the latter being taken as a contrast to the ~~native~~ mature bare-seeded plants, as it was an important wood which had been extensively studied.

The grass samples were cut just above ground level, with scissors, but it was easier to lift each individual clover plant intact and then cut off the roots. In the case of the grasses, each individual tiller was counted as a separate plant. From 50 - 200 plants (according to the size) were taken to determine the dry matter per 100 plants, and a further

250/

250 - 500g. were oven dried at 100°C and finely ground for analysis. Additional samples were chopped, dehydrated with alcohol and dried at 50°C (2 days). In the case of immature lucerne, the main sample of the fresh material was minced, washed with water, dehydrated with alcohol and extracted directly with ethanol-benzene.

Lignin determinations were made by the final procedures described in Chapter VII.

8.2 Results and discussion.

8.2 (i) Percentage of total 'true' lignin.

The total true lignin as a percentage of the dry matter increased with advancing age, the range between young and mature plants being distinctly narrower with the legumes than with the gramines as the mature gramines contained 1½ to twice as much lignin as the mature legumes (Table A41). An increase in the lignin was associated with a decrease in protein but the figures were too few to show a significant correlation.. All samples of plants at a young stage of growth were low in lignin, containing between 1 and 2.7% in the dry matter. The two samples of young clover (Treatments 1a+ & 1a, Table A41) were of the same age but the one taken later in the season (1a +) was shorter (the dry matter per 1,000 plants was considerably lower,) and had a lower lignin content. The very young timothy, although of the same age as the very/

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very young Italian ryegrass, (Sample 2a), was only growing slowly and contained a higher lignin content, similar to that of the Cocksfoot of the same age (3a) which was growing vigorously (Length of 5cm.) and was probably more physiologically mature. There appeared to be differences between the individual species of gramines; Italian ryegrass straw was relatively low in lignin content, but oat straw contained the highest percentage. In legumes the lignin content appeared to increase gradually from the young to the mature stage, although this may have been due to the relatively small total difference between young and mature plants. In the gramines, however, the lignin percentage seemed to rise gradually in the early stages before flowering, but rose markedly in the samples examined after flowering. Thus, the lower digestibility observed with mature legumes, such as lucerne, cannot be attributed to the lignin percentage which is distinctly lower than in mature gramines.

Armstrong et al., (1950) have recently published the following figures for lucerne and cocksfoot at various stages of growth, the lignin being determined by the Norman-Jenkins procedure but corrected for crude protein.

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		% of Original Dry Material	
		Crude Protein	Corrected Lignin
Lucerne	cut 16/4/48	35.91	3.63
	26/5/48	23.11	6.70
	7/7/48	18.90	7.17
Cocksfoot	cut 16/4/48	14.93	5.50
	26/5/48	8.51	6.09
	9/7/48	5.78	9.74

Using the protein figures as an indication of stage of maturity, comparison of the above lignin data with those for 'true' lignin in Table A41 shows that with lucerne the Norman-Jenkins lignin, corrected for protein, is higher at all stages of growth. In the case of cocksfoot, however, figures of Armstrong et al (1950) ~~figures~~ are higher for the young material, similar in the case of the plants of intermediate maturity and lower for the mature plants. This is because of the yield of Norman-Jenkins lignin (ash-free or corrected for protein) being affected by two factors acting in opposite directions, (a) losses due to hydrolysis with dilute acid in the pretreatment and also in the final treatment of the wet lignin residue, (b) gains due to the presence of contaminants, insoluble in 72% H_2SO_4 or precipitated during dilution of the strong acid, or/

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or contaminants which condense with the true lignin. With plant material containing appreciable amounts of nitrogen (legumes at all stages and gramines up to the flowering stage) the gains appear to exceed the losses, the difference between 'true' lignin and Norman-Jenkins lignin being greater when the nitrogen content of the plant is highest. Although correction for crude protein reduces the apparent gain in the lignin fraction, the presence of nitrogenous material is always associated with contamination by non-nitrogenous material, and the gains in corrected lignin still exceed the losses. With plant material of low nitrogen content, on the other hand (mature gramines) the losses of the lignin fraction exceed the gains from contamination, so the Norman-Jenkins procedure gives low results. The effect is shown in Table A42 where the proposed method is compared, not with the Norman-Jenkins method, but with the similar current procedures of Ellis et al (modified) and Armitage et al (modified). With the young clover and immature pasture, containing high nitrogen contents, the Ellis or Armitage figures for corrected lignin were 50% to 100% greater than the 'true' lignin determined by the proposed method, but in the case of timothy hay, containing

much/

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much less nitrogen, although the Ellis lignin (corrected) was higher than the 'true' lignin, the difference was only 10%. With gramines beyond the hay stage, similar figures for Ellis lignin (corrected) and 'true' lignin would be expected; and with very mature material, the losses involved in the current methods would exceed the contamination and lead to results lower than the 'true' lignin as was indicated above by comparison of the results for mature Cocksfoot in Table A41 with those for comparable material examined by Armstrong et al., (1950).

From Table A42, it is also obvious that the lignin fractions obtained by current methods of analysis will always contain lower amounts of 'true' lignin than the fraction obtained by the proposed method. Moreover, the lignin fractions obtained by different current methods (e.g. Ellis and Armitage) will vary in quantity and quality according to the pretreatment and dilute acid hydrolysis, which govern the losses of lignin, and the gains due to contamination. With young clover the Armitage procedure gave a lower yield of lignin than the Ellis method, because in the former the pretreatment was more drastic. The Armitage lignin was also purer than both the Ellis lignin and the fractions obtained by the proposed method, although it did not contain the

whole/

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whole of the 'true' lignin as indicated by the results of the proposed method.

Only in relatively mature gramines with low nitrogen percentage will the lignin figures obtained (ash-free or corrected) by current methods be similar to the 'true' lignin obtained by the proposed method; in rare cases the results may be identical if the losses of lignin in the current methods are balanced by the gains due to contamination. In very mature gramines the losses may exceed the gains and result in a lignin figure lower than the 'true' lignin. Most of the current methods of lignin determination agree reasonably closely with mature materials, such as straws, but differ very widely with immature and young material (McDougal and De Long, 1948 C). For the latter, the current methods give only approximate figures for lignin by comparison with the 'true' lignin obtained by the proposed method. Since wood resembles the gramine straws, the gains and losses involved in the usual methods approximately balance one another, which accounts for the 'true' lignin figure (23.57%) obtained for Scots Pine, (Table A41) being comparable with the figures for ash-free acid lignin recorded in the literature, (Freeman, 1946, P.663) reported 26.1% in the dry matter of alcohol-extracted Scots Pine).

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8.2 (ii) The absolute content of 'true' lignin per 1000 plants

Changes in percentage composition are important in determining the feeding value of a plant, but to show the changes actually taking place in the plant during growth it is necessary to consider figures for the absolute composition rather than the percentage composition. As Phillips et al., (1939) realised the percentage composition of a plant at different stages of development may be misleading, as growth is a dynamic process and an increase in the percentage of one component does not necessarily indicate an actual increase in the absolute amount of that component; it may well be due to a diminution or translocation of one or more other components. In table A41 the absolute amounts of dry matter, crude protein and 'true' lignin are expressed as g. per 1000 plants.

The absolute true lignin increased as the plants grew, but the rate of increase was greatest at the beginning in the case of clover and cocksfoot, though more uniform in the case of Italian ryegrass as shown by the ratios below

(See over for table)

In the case of clover the growth between stages a and b was faster than between b and c, as indicated by the dry matter ratios and the rate of lignin formation was/

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	Clover			Italian Ryegrass			Cocksfoot		
	D.M.	T.L.	C.P.	D.M.	T.L.	C.P.	D.M.	T.L.	C.P.
g. in sample b / g. in sample a	4.5	11	3.0	1.6	1.8	1.5	2.1	3.2	1.5
g. in sample c / g. in sample b	1.2	1.7	1.4	1.1	1.7	1.1	1.4	1.7	1.2

was faster than the rates of growth, or of crude protein formation. In the case of cocksfoot and Italian ryegrass however, although the rate of lignin formation was also higher than that of dry matter or crude protein, between stages a and b, the difference was not nearly so marked as was the case with clover. It may be that young legumes, whose plant stems are formed at the beginning of growth, need more rapid lignin formation for their support, whereas gramines which form their stems later, do not need such rapid lignin production in the early stages. Whereas in clover the absolute amount of crude protein was greater than the absolute amount of 'true lignin' at all stages of growth (but particularly in the young plants) in Timothy the absolute protein exceeded the lignin in the young plants but the absolute lignin was greater than the protein in the mature growth.

8.2 (iii) Relation between soluble and insoluble lignin fractions.

It was observed that the gramines differed characteristically from the legumes in the relative amounts of soluble/

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soluble lignin present in the total lignin. This is indicated by the absolute methoxyl present in the soluble, insoluble and total lignin fractions. In the mature and immature lucerne the soluble lignin formed only 17 to 20% of the total lignin and the corresponding proportion for clover at all stages of growth was about 27%, but in the gramines the proportion was 50% or more, being as high as 71% in young timothy and 61% in young oats. In immature grass (containing 37% clover) the soluble lignin was only 48% of the total lignin, no doubt because of the presence of a considerable proportion of clover. This difference may prove to be useful in differentiating between these two main types of animal foods. In Timothy which was examined at three different stages of growth, the proportion of soluble lignin was greatest (71%) in the young plants, less in the hay (51%) and still less (47%) in the straw, so that changes in the proportion of soluble lignin may characterise the stage of maturity in non-legumes.

In Scots pine, the soluble lignin was only 1.4% of the total lignin and the marked differences between softwood, legumes and gramines, are probably due to differences in the physical properties of their lignins, possibly the result of differences in the degree of polymerisation in the lignin molecules. In Italian ryegrass straw 60% of the total lignin

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was soluble in 72% H_2SO_4, and it is possible that other gramine straws may contain lignin even more soluble; this may explain why Hilpert and Littmann (1935) claimed almost complete solubility of a straw in 72% H_2SO_4 under certain conditions (-12°C) although their results could not be repeated by other workers (Chap. III)

There was a high proportion of the insoluble lignin fraction in legumes and the percentage of this fraction was much higher in young legumes than in young gramines, although in mature legumes and mature gramines the percentages were similar. The low digestibility of mature legumes may possibly be attributed to the amount of insoluble lignin rather than the total lignin, and this may also be true in the different gramine straws. Mature lucerne contained a higher percentage of insoluble lignin than mature clover, and Italian ryegrass straw contained a distinctly lower percentage of insoluble lignin.

Of the mature gramine straw examined, Italian ryegrass contained the least insoluble 'true' lignin (3.39%), Cocksfoot contained 4.92%, oats 5.51% and timothy 5.93% and these may be related to their digestibilities for Schneider's[5] data (1947) show the 'total digestible nutrients' in ryegrass straw to be higher than in oat straw (57.1% of the dry matter in the former and 44 to 48% in the latter) and the data/

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data for mature hays suggest that Italian ryegrass is the most digestible and timothy the least digestible.

It is also significant that the yield of alkali lignin obtained from legumes was always much less than that obtained from gramines, probably because the lignin fraction of gramines soluble in 72% H_2SO_4 was also easily soluble in alkali. It was realised by previous workers (Norman, 1937 p.154, Phillips, 1946 p.287) that the lignin in the straws of gramines is more easily extractable by alkali than that in wood, and this may be explained by the proportion of the lignin soluble in 72% H_2SO_4.

The findings of Armstrong et al. (1950) that the crude fibre of legumes had a much higher lignin content than that of gramines, and that the recovery of lignin in the crude fibre (relative to that in the original plant) is greater in legumes may also be explained by the resistance of legume lignin to the alkali extraction used in crude fibre preparation, this resistance being due to the physical nature of the legume lignin and the lower proportion of the soluble lignin fraction.

C H A P T E R IX

The Digestibility of Lignin and its Significance in Animal Nutrition Studies.

The proposed method for lignin determination (Chap. VII) was tested in feeding experiments in order to examine separately the digestibilities of the two distinct fractions designated 'soluble true lignin' and 'insoluble true lignin' respectively. It has already been noted (Chap. VII) that the proportion of soluble fraction in the total lignin from faeces was less than the proportion in the total lignin from the food, and appreciable digestibility of the soluble fraction appeared to be indicated.

9.1 Experimental

Two digestibility trials were carried out, the first with chopped timothy hay fed to two sheep and, the second with fresh immature autumn grass (cut daily in September 1950 from Kimmerly Hill, Boghall Farm) fed to four sheep.

The timothy hay was fed to two 1 year old half-bred wethers, A and B and the faeces collected daily but bulked in four day periods for each sheep. With sheep A faeces were collected for four consecutive periods (16 days) and with sheep B for 3 periods (12 days). In all cases faeces collections were made 24 hours after the feeding and an adequate preliminary period/

period was allowed before commencing experiments. Samples obtained in period I with sheep B and dried at 100°C were used for a preliminary test of lignin digestion (Section 2), and also for studies on the lignin determination procedure already described. (Chap. VII).

The immature pasture grass contained 37% clover (on a dry matter basis) and was fed to three 6 months old Oxford-cross wethers and one 18 months old Half-bred wether. It was cut by scythe each morning, then chopped and mixed before feeding, in order to facilitate sampling and minimise selection by the animals; three to five Kg. of the fresh grass was fed to each sheep daily. The sheep had previously been out at pasture and were fed uncontrolled amounts of fresh grass when they were first transferred to the experimental cages; after settling down the grass feeds were adjusted to the level to be employed in the experiment and after a preliminary period of four days faeces were collected for 12 days. Owing to the variable weather conditions the moisture content of the herbage varied from 16 to 24.9% and there were considerable fluctuations in the daily dry matter intake and in the faecal dry matter excreted. In this experiment the food and faeces samples were not bulked in 4 day periods but each was dried separately at 100°/

100°C. In addition separate daily samples of food and faeces were dehydrated with alcohol then dried at 50°C (2 days). At the end of the experiment 12 day composite samples of dried food and faeces were prepared for each sheep.

Determinations of soluble and insoluble lignin were made by the final method described in Chapter VII except in the preliminary test with the period I samples from sheep B, when proposed methods 1 and 2 (without final hydrolysis) were employed. In the calculation of true lignin, methoxyl conversion factors of 5.52 for timothy hay and 5.46 for the immature grass, were used. Ellis lignin was prepared by the modified procedure given in the Appendix (p12) and other nutrients were determined by the usual methods. 'Crude carbohydrate' was obtained by subtracting from 100 the sum of the percentages (in the dry matter) of ether extract, crude protein, true lignin and ash.

9.2 Preliminary test of lignin digestibility

The results obtained with sheep B in Period I of the Timothy hay experiment (Table A43) indicated that the insoluble true lignin (No.2b) was almost completely recovered in the faeces and so was practically indigestible, whereas the soluble true lignin was digested to a marked extent (Ca 30%) The results/

results suggested that the insoluble true lignin might possibly be used as a natural marker, and enable the digestibilities of other nutrients to be computed from their different ratios with lignin in feed and faeces. Clearly the total lignin figures could not be used in this way. Although the ash-free or corrected Ellis lignin (Treatment No. 1) was of low digestibility it was higher than that of the insoluble true lignin (Treatment No. 2b). The digestion of the true lignin present in the Ellis lignin fraction (1c) was higher still, as the Ellis lignin from the faeces (containing 2.41% N and 10.22% MeO) was more contaminated than that from the food (containing 2.42% N and 11.48% MeO). As the Ellis lignin fraction contained 80.9% of the total true lignin, (As indicated by Treatment No. 2a) it would contain a fraction of the soluble true lignin, and thus be of a higher digestibility than the insoluble true lignin.

It was also clear that using the ash-free or corrected Ellis lignin figures, the digestibility would be affected by the differences in contamination between faeces lignin and food lignin. In this particular case (Table A43, Treatment No. 1) the faeces lignin was slightly more contaminated than the food lignin and so reduced the digestibility

from/

from 13.91% to just over 9%, and still greater contamination of the faecal lignin would have resulted in a still lower or even negative digestibility besides increasing the variations between results. Doubts of the validity of using Ellis lignin as a natural marker and reference material for digestibility measurements have been indicated recently by Forbes & Garrigus (1950 b), no doubt due to the variable degree of contamination of the lignin obtained as well as to the inclusion of some lignin fraction with appreciable digestibility. They found that the yield of ash-free lignin could not be accurately duplicated although the absolute methoxyl present was practically constant, illustrating the danger of relying on figures for ash-free lignin and the greater reliability of data based on the absolute methoxyl present. These workers obtained significant negative digestibilities of the Ellis lignin, undoubtedly because (in all but one case) the faeces lignin was more contaminated with nitrogenous material.

The appreciable digestion of the soluble true lignin indicated in Table A43 (Treatment No. 2c) explained the finding of a lower proportion of soluble lignin in the total lignin of the faeces than in the food.

9.3 Digestibility of True Lignin.

The results obtained in the feeding of Timothy hay (Table A45) and immature pasture (Table A47) indicated a significant digestibility of the total true lignin and the soluble true lignin, but no significant digestion of the insoluble true lignin. The mean digestibility of the insoluble lignin in pasture was - 4.31% ± 3.69% which did not differ significantly from zero ($P > 0.2$) and the corresponding value with Timothy hay was - 0.1% ± 3.6%, which was obviously not significantly different from zero ($P > 0.5$). Analysis of the alcohol dehydrated samples of pasture grass and faeces gave digestibilities for insoluble true lignin of - 2.7%, - 3.4%, + 9.6% and - 6.8% for sheep H. J. K. and L. respectively, the mean (0.8% ± 3.59%) being again not significantly different from zero or from the average digestibility calculated from the analyses of oven dried samples.

The soluble true lignin in immature pasture was almost twice as digestible as that in Timothy hay, and this led to a higher digestibility of the toal true lignin in the pasture. The digestibility of the soluble true lignin in pasture was of a relatively high order, (41.21%) and it appears that the digestibility of this fraction may decrease with maturity as

is/

is generally the case with other plant nutrients.

The digestion of the soluble true lignin indicated that this fraction was more susceptible to biological treatment in the digestive tract as well as to chemical treatment as indicated by its observed solubility in 72% H_2SO_4 and conc. HCl. It seems highly improbable that this fraction of the lignin was partly dissolved by the alkaline condition of the intestine and absorbed as such, but more probable that it was split by some specific enzyme present in the digestive tract. In "Vitro" digestion of seeds-hay with trypsin - Na_2CO_3 hardly reduced the absolute methoxyl content of the ethanol-benzene-extracted material (Table A 8) or the absolute methoxyl in the acid lignin fraction (Table A 27). Moreover, warm 0.5N-NaoH extracted negligible amounts of alkali lignin from air dried young clover (Table A 14).

The digestibility of the total true lignin was low, being only 10.8% in the case of timothy hay and 17.3% with pasture, corresponding to only 0.96% and 0.62% of digestible true lignin in the dry matters of the hay and pasture respectively. In no type of fodder is the percentage of digestible true lignin likely to be much higher than the figures quoted for hay and grass; in very mature material the higher lignin/

lignin content would be offset by lower digestibility, whilst with very young material, a possibly higher digestibility would be negatived by a lower percentage of lignin present. Although lignin has a relatively high calorific value (6.277 Cals. per g. according to Rubner, 1928) it will be of little use as a source of energy to the animal. The animal body can only tolerate limited amounts of aromatic substances, so the small amounts of digestible lignin present in foods is probably fortunate.

The amounts of aromatic amino acids provided by the digestible proteins in foods are also not of a high order; in the dry matter of young grass containing 20% digestible crude protein, the digestible aromatic amino acids would form about 2% (the aromatic amino acids forming about 10 to 13% of the plant proteins according to Hawk et al., 1949, P 108). The results of Phillips et al (1929) and Csonka et al (1929) (See Tables 2&3 P102-3) showed that alkali lignin could be digested by dogs and the increased urinary excretion of hippuric acid indicated the limited tolerance of the animals for the digested aromatic material. Further investigation of the intermediary metabolism of lignin in the body is needed, and it may show an important relation between lignin/

lignin and the aromatic amino acids, particularly the phenolic ones; the three carbon side chains in the aromatic nucleus of lignin (syringyl or guaiacyl radicals, (XXXVII, XXXIX, Appendix P 30) might be oxidised in the animal body, and the aromatic nucleus may be of use to the animal if it cannot synthesise this group.

The digestibility of the Ellis lignin of pasture grass by sheep H is shown in Table A48, the figure for the true lignin in the Ellis lignin fraction (10.29%) being intermediate between the digestibility of the total true lignin (20.33%) and of the insoluble true lignin (-6.24%) as was also the case with timothy hay (Table A43). Unlike the results with hay, however, the Ellis corrected lignin and ash-free lignin (containing 4.44%N and 6.61% MeO) present in pasture had higher digestibilities than the true lignin in the Ellis lignin fraction, because in this case the Ellis faecal lignin (containing 3.91%N and 7.22% MeO) was less contaminated than the food lignin. The unreliability of results based on corrected or ash-free lignin figures was thus again confirmed. The true lignin in the Ellis lignin was 83% of the total true lignin in the case of the food, but 93% in the case of the faeces, the higher percentage for faeces being no doubt due to the greater amount of the

insoluble/

insoluble true lignin which appeared to be more resistant to the dilute acid hydrolysis used in the Ellis procedure.

The 'crude carbohydrate' in timothy hay (Table A45) was of higher digestibility than either the crude fibre or the nitrogen-free extractives, undoubtedly because of the separation of the poorly digested true lignin fraction. Thus it is more biologically sound to separate the lignin fraction as in the proposed scheme of analysis. The higher digestibility of the 'crude fibre' than of the nitrogen-free extract, was most probably due to presence in the latter of a much higher amount of true lignin than in the former. Previous results (Tables A17 & A27) with seeds-hay sample 1 (predominantly Italian ryegrass) showed that when subjected to 8 hours alkali extraction (0.5N -NaoH, i.e. Ca 2% NaoH) at 85°C, lost about 87% of its lignin (indicated by the fall in absolute methoxyl), and it is to be expected that in the routine boiling with 1.25% NaoH used to prepare crude fibre, the greater part of the lignin will be removed. This is probably why, in gramines, the crude fibre digestibility is found to be about equal to, or greater than, the digestibility of nitrogen-free extractives. In legumes, the crude fibre is of lower digestibility than the nitrogen-

free/

free extractives, probably due to the presence of a higher true lignin content in the former, as legume lignin appears to be naturally resistant to alkali extraction. The presence of lignin in the crude fibre fraction may be of more importance than the amount of cellulose in determining the digestibility, particularly with ruminants, but more determinations of 'true lignin' in food, faeces, and crude fibre fractions in digestibility trials with gramines and legumes are necessary to confirm these deductions.

The proposed scheme of analysis would enable the calculation of the total digestible nutrients, possibly neglecting the digestible lignin or giving it the same value as digestible carbohydrate until its metabolism in the body is determined. Difficulties would arise, however, in calculating the starch equivalent of the food, by Kellner's method which requires a 'crude fibre' deduction in the case of roughages.

9.4 The lignin ratio technique for determining the digestibility of nutrients.

The digestibility of insoluble true lignin indicated that it could be used as a reference material to calculate digestibilities indirectly. This was tested/

tested by calculating the digestibility of nutrients by a formula similar to that given on page 95 Text, used by Hale et al (1947).

In both the hay and pasture experiments (Tables A45 and A47) there was no significant difference between the average digestibilities calculated by the conventional method and those obtained from the lignin ratio. This was inevitable as the average digestibility of the insoluble lignin fraction was not significantly different from zero. It was also observed in the case of timothy hay that although the average digestibilities obtained by the two methods were practically identical, the standard errors of the means obtained from lignin ratio digestibilities were greater than the standard errors of the means obtained by the conventional method. This was because the small positive digestibility of insoluble true lignin by sheep A (+ 3.5%) lowered the calculated lignin ratio digestibilities of each nutrient, whereas with sheep B, the negative digestibility of insoluble true lignin (- 3.6%) increased the calculated lignin ratio digestibilities, so that the combined lignin ratio digestibilities for the two sheep showed much greater variability.

With pasture grass, however, the standard errors of the means obtained from lignin ratio digestibilities

were/

were greater than the standard errors of the means calculated by the conventional method for only four out of the seven nutrients examined, so that variations in digestibilities calculated by the two methods were, in this case, similar.

It was noticeable in both trials that the greatest variability was found in the digestibility of the insoluble true lignin, the range with timothy hay being from -3.6 to +3.5, and with pasture from -10.41 to +6.43. In order to examine further the association of the greatest variability with the least digestible nutrient, it was considered necessary to study the mathematical relationships between the conventional and lignin ratio methods. It was also observed that the effect of slight digestion of the 'insoluble true lignin', on the lignin ratio calculated digestibility varied in magnitude from one nutrient to another, the effect being greatest on the fractions of lowest digestibility. Thus, with sheep L the -10.41% digestibility of the insoluble true lignin (Table A47) increased the relatively high crude protein digestibility from 75.43% (conventional) to 77.75%, (lignin ratio) ~~(2. 3% higher)~~ this being an increase of only about 3%. On the other hand the low digestibility of total true lignin was increased from 10.98% (conventional) to 19.39% (lignin ratio),

this/

this being an increase of about 76%.

In the case of Ellis lignin with sheep H (Table A 48 and Fig. 15 p.40 Appx.) there were great differences between the conventional digestibilities and ~~those obtained~~ by lignin ratio calculated digestibilities, particularly where the ash-free lignin figures were used, since this fraction had a digestibility of 20.6%; the nutrients with low digestibility (conventional) were seriously affected.

9.5 Mathematical relations between conventional and lignin ratio methods for calculating nutrient digestibilities.

9.5 (i) Calculation of digestibilities by the conventional method (D).

The calculation of the apparent digestibility of a given nutrient may be obtained by the formula on the following page.

The fraction $\left(\frac{\text{g. dry matter in faeces voided}}{\text{g. dry matter in food eaten}}\right)$ may be designated the 'dry matter ratio', (W) and the second fraction $\frac{\%\text{ nutrient in dry faeces}}{\%\text{ nutrient in dry food}} \times 100$ may be designated the 'nutrient ratio' (N), so that the digestibility obtained by the conventional method (D) as given by the formula :- $D = 100 - WN$. This equation may be represented by a straight line curve (Fig. 9 p.40 Appx.) prepared by using the value of W obtained with any one animal in a single experiment and (Equation 1)

plotting/

EQUATION 1.

Digestibility (D)

$$= 100X \frac{\text{G.Dry Matter consumed X\% nutrient in dry food} - \text{G.Dry Faeces voided X\% nutrient in dry faeces}}{\text{G.Dry Matter consumed X\% nutrient in dry food}}$$

$$= 100 \left(1 - \frac{\text{G.Dry Faeces voided X\% nutrient in dry faeces}}{\text{G.Dry Matter consumed X\% nutrient in dry food}}\right)$$

$$= 100 - \frac{\text{g.dry matter in faeces}}{\text{g.dry matter consumed}} \times \frac{\text{\% nutrient in dry faeces}}{\text{\% nutrient in dry food}} \times 100$$

$$= 100 - WH$$

plotting the values of N corresponding to two values of D (e.g. for D = 0 & 100).

An equation or curve of this sort facilitates the calculation of nutrient digestibilities since, once the value of W has been determined for the particular experiment, only the nutrient ratios need be calculated for each nutrient digestibility required.

9.5 (ii) Calculation of digestibilities by the lignin ratio method (D_1.)

The apparent digestibility determined by the lignin ratio technique (D_1) is given by the formula

$$D_1 = 100 - \frac{\%\text{ lignin in dry food}}{\%\text{ lignin in dry faeces}} \times \frac{\%\text{ nutrient in dry faeces}}{\%\text{ nutrient in dry food}} \times 100$$

If $\frac{\%\text{ lignin in dry food}}{\%\text{ lignin in dry faeces}}$ is designated 'lignin ratio' and represented by the symbol L, the formula becomes :

$$D_1 = 100 - L \times N \qquad \text{(Equation 2)}$$

When lignin digestion is zero, $D_1 = D$ and $L = W$.

9.5 (iii) Comparison of the two methods of calculating digestibilities.

Figs. 9 to 14 show the graphs corresponding to equations 1 (conventional) and 2 (lignin ratio) for the six sheep digestibility experiments.

Figure 9 represents the digestibility of timothy hay by sheep A, when the insoluble true lignin was digested to the extent of 3.5% (Table A45) and the following observations are based on this figure and the/

the two equations.

(1) The two curves coincide when the digestibility is 100%, and the lower the digestibility the greater is their divergence. Thus, at low levels of digestibility the digestibility percentage calculated by conventional and lignin ratio methods will show the greatest divergence, as already observed.

(2) Except when digestibility is 100%, D_1 will always be less than D, and the difference, expressed as a proportion of D_3 ($(D-D_1) \div D$) will increase as digestibility falls.

(3) When D = 100, N = 0 and when D = 0, N = 100/W so that two points for the construction of the graphs are readily obtained.

(4) When N/100 is greater than 1/W the value of D is negative, and this may arise when metabolic excretions lead to a greater quantity of a particular nutrient in the faeces than in the food consumed. A value of D greater than 100 is, however, impossible as this would necessitate a negative value for W or N.

9.5 (iv) Relation between D and D_1.

$$\text{For lignin } N/100 = \frac{\% \text{ lignin in dry faeces}}{\% \text{ lignin in dry food}} = 1/L$$

$$\text{Digestibility of lignin } (G) = 100 - W \times \frac{100}{L} \quad \text{i.e.}$$

$$G/100 = 1 - W/L \quad (3). \qquad \text{(Equation 3)}$$

From/

From equation (1) $\overline{D} = 100 - WN$ $W = (100 - D)/N$ and from equation (2) $\overline{D}_1 = 100 - LN$ $L = (100 - D_1)/N$

$$\text{So } W/L = \frac{100 - D}{100 - D_1}$$

Substituting in equation (3) -

$$G/100 = 1 - \frac{100 - D}{100 - D_1} = \frac{100 - D_1 - 100 + D}{100 - D_1} = \frac{D - D_1}{100 - D_1}$$

Thus, $G(100 - D_1) = 100\ (D - D_1)$ i.e. $100G - D_1 G = 100D - 100D_1$

i.e. $D_1 (100 - G) = 100 (D - G)$ or $D = (100G + 100D_1 - D_1 G)/100$

i.e. $D_1 = \frac{100\ D}{100 - G} - \frac{100\ G}{100 - G}$ or $D = G + D_1 (\frac{100 - G}{100})$

Thus, if the digestibility of the lignin is known the digestibility of any nutrient calculated by the conventional method may readily be converted into the corresponding digestibility by lignin ratio and vice versa.

9.6 Calculation of Total Digestible Nutrients (T.D.N.)

From equation (1) $\overline{D} = 100 - WN$ it may be deduced that percentage digestible nutrient in food =

% nutrient in food x % digestibility/100

= % nutrient in food (1 - WN/100)

= % nutrient in food $(1 - W \times \frac{\%\text{ nutrient in faeces}}{\%\text{ nutrient in food}})$

= % nutrient in food - W x % nutrient in faeces (Equation 4)

Similarly from equation (2) $\overline{D}_1 = 100 - LN$ it may be deduced that percentage digestible nutrient in food = % nutrient in food - L x % nutrient in faeces. (Equation 5)

Total digestible nutrients may therefore be calculated from dry matter (W) or lignin (L) ratios in the following manner.

Let O = % organic matter in food and

O_1 = % organic matter in faeces and

Let F = % fat in food and F_1 = % fat in faeces

Then T.D.N. = % digestible protein + % digestible carbohydrates + 2.25 x % digestible fat

= % digestible organic matter + 1.25 x % digestible fat

$= (O - LO_1) + 1.25 (F - LF_1)$ (from equation (5))

$= (O - WO_1) + 1.25 (F - WF_1)$ (from equation (4))

9.7 Effect on apparent lignin digestibility of errors in faeces collection or lignin determination.

The digestibility of lignin (G) is indicated by equation (3) $G/100 = 1 - W/L$

i.e. $G = 100 - 100\ W/L$

If the digestibility is nil when there are no errors,

$G = 0$ and $W/L = 1$ i.e. $W=L$

An error of +5% in the weight of faeces will produce an error of +5% in the value of W, but L will not be affected. The apparent digestibility of lignin (G_2) will then be given by $G_2 = 100 - 105\ W/L = -5$.

An/

An error of +5% in the determined percentage of lignin in the food will produce an error of +5% in the value of L but W will not be affected. The apparent digestibility of lignin (G_3) will then be given by

$$G_3 = 100 - 100W/1.05L$$
$$= 100 - 100W/1.05W$$
$$= 5/1.05$$
$$= 4.76$$

The combination of an error of +5% in faeces weight with an error of +5% in the food lignin percentage will give an apparent lignin digestibility (G_4) as follows.

$$G_4 = 100 - 105W/1.05L = 0.$$

Thus, the two errors in this case cancel out.

The combination of an error of +5% in faeces weight with an error of -5% in the food lignin percentage will give an apparent lignin digestibility (G_5) as follows.

$$G_5 = 100 - 105W/0.95L = (95 - 105)/0.95$$
$$= -10/0.95$$
$$= -10.5$$

In this case the two errors are cumulative.

Thus, although the insoluble true lignin may be completely indigestible, it is possible to get positive or negative digestibilities as a result of errors in faeces collection or lignin determination

and,/

and, in some cases, these errors may be cumulative and so suggest quite appreciable digestibility. When comparing the lignin ratio technique with the conventional procedure it is, therefore, desirable to use as many animals as possible in order to minimise the errors.

9.8 The lignin ratio correction of Hales et al (1947)

These workers claimed that in using the lignin ratio technique, correction could be made for discrepancies arising from some digestion of the lignin (P 99 Chapt.I). They used, both the conventional scheme of analysis, (into crude fibre and N-free extractives) and that of Crampton & Maynard (1938) (with subdivision into cellulose, lignin and "other carbohydrates") and by the lignin ratio method calculated digestible crude fibre + digestible N-free extractives (A) as well as digestible cellulose + digestible "other carbohydrates" (B). They then assumed that the difference between these two values (A - B) would equal digestible lignin, and proceeded to make a correction for it.

It may be shown, however, that this assumption was incorrect, and that the value of (A - B) should be zero.

For convenience, the percentages of the different nutrient fractions in food and faeces are designated

by/

by letters as follows :-

		% in food	% in faeces
Crude fibre	=	R	R_1
N-free extract		E	E_1
Cellulose		U	U_1
Lignin		I	I_1
"Other carbohydrates"		H	H_1

Then $R + E = U + I + H$ (6) (Equation 6)

$R_1 + E_1 = U_1 + I_1 + H_1$ (7) (Equation 7)

and $L = \frac{I}{I_1}$ (8) (Equation 8)

From equations (5) (Page 408) and (8) we get

% digestible nutrient in food = % nutrient in food $- \frac{I}{I_1}$ x % nutrient in faeces

and using this formula the following values are obtained.

A = Digestible crude fibre + digestible N-free extractives.

$= R - \frac{I}{I_1}.R_1 + E - \frac{I}{I_1}.E_1 = (R + E) - {}^{I}/_{I_1}\,(R_1 + E_1)$

B = Digestible cellulose + digestible "other carbohydrates".

$= U - {}^{I}/_{I_1}.U_1 + H - {}^{I}/_{I_1}.H_1$

$= (U + H) - {}^{I}/_{I_1}\,(U_1 + H_1)$

$= (R + E + I) - {}^{I}/_{I_1}\,(R_1 + E_1 + I_1)$
(from equations (6) and (7)).

$= R + E - {}^{I}/_{I_1}\,(R_1 + E_1 - I_1 + I.I_1/I)$

$= R + E - {}^{I}/_{I_1}\,(R_1 + E_1)$

= A.

Thus./

Thus, since B = A, A-B = 0 and the claim of Hales et al to have obtained a value for digestible lignin from the difference A-B must clearly have resulted from some miscalculation. Indeed, the nil value for digestible lignin is also clearly shown by applying equations (5) and (8) to lignin, giving -

$$\% \text{ digestible lignin} = I - {}^{I}/_{I_1} \cdot I_1 = 0.$$

Since the lignin ratio technique is based on the assumption of complete indigestibility of lignin, this assumption also applies to any derived values or equations and makes it impossible to determine digestible lignin in any way such as that suggested by Hales et al.

9.9 The application of the lignin ratio method in practice.

Several digestibility experiments need to be made to confirm that the recovery of the insoluble true lignin is 100%, and in order to obtain a reliable figure for the dry matter ratio (W) the experimental period should be fairly long. The experiment with pasture grass showed that there was not a high correlation between the daily dry matter consumption and the daily faecal dry matter excretion (Table A46), most probably due to the fluctuating moisture content of the food and the consequential variations in daily dry matter intake.

If/

If the recovery of insoluble true lignin proves to be complete, then the lignin ratio may be used to check the digestibilities obtained by the conventional method, and to obtain digestibilities without quantitative collections of food and faeces, particularly with foods of relatively constant composition, such as hay. With grass-feeding, however, further examination of the day to day fluctuations in the lignin percentages of food and faeces is needed to enable lignin ratio digestibility experiments to be properly designed and to permit reasonable estimates of the dry matter intake of grazing animals.

General Summary

Various preparations of acid lignin, alkali lignin and ester lignin were made from plant materials and faeces in order to study their yield and purity and their possible use as a basis of lignin determination. The method of determination finally adopted was used to study the lignin content of various plants and its significance in nutritional studies.

Examination of the solubility of lignin in 72% H_2SO_4 did not confirm Hilpert's claim that lignin is an artefact.

Reproducibility studies indicated the presence of analytical errors in the current methods of determining lignin and the various residues obtained at different stages of the lignin determination were therefore studied. Their origination from lignin indicated the necessity to avoid the losses incurred by dilute acid prehydrolysis and in the final filtrations.

A similarity between acid lignin and alkali lignin was established and in both lignins there were analogous fractions soluble and insoluble in 72% H_2SO_4. The finding of a fraction of the acid lignin of feeding stuffs which was soluble in 72% H_2SO_4 was contrary/

contrary to the general belief that lignin is completely insoluble in strong acids.

The ash and nitrogenous material in lignin fractions were found to be contaminants, and contamination with carbohydrates was also invariably present in acid lignin preparations. The application of a protein correction based on the usual factor of 6.25 appeared to be a fairly satisfactory way of dealing with the nitrogenous contamination of acid lignin fractions but the presence of protein was invariably associated with carbohydrates contamination for which there was no satisfactory means of correction.

The presence of unpredictable amounts of contaminants in acid lignin (even in acid lignin derived from alkali pulp) and the losses of lignin occurring during dilute acid hydrolysis rendered acid lignin figures (either ash-free or corrected for protein) unreliable.

Alkali lignin from mature gramines may be sufficiently pure to serve as a reliable reference lignin but that from legumes did not prove suitable.

Evidence was obtained indicating the reliability of the absolute methoxyl content of acid lignin fractions as a measure of the true lignin present, if the procedure avoided losses of lignin but removed contaminants/

contaminants containing methoxyl.

The ester lignin prepared from the alkali pulp of mature plants was found to be reliable reference lignin; and it was found that the methoxyl content of this lignin was relatively constant in different plant species, indicating that apparent variations are due to contamination.

The final method was based on the absolute methoxyl content of the acid lignin fraction, the true lignin being calculated with a methoxyl conversion factor derived from the methoxyl content of the reference (ester) lignin prepared from the mature plant. There was evidence that a methoxyl conversion factor of 5.50 might be a reliable average for feeding stuffs, but it is preferred to use an individual factor for each plant until further data are available for a greater number of plants.

There was a characteristic difference between legume lignin and gramine lignin towards extraction with alkali, and also in the relative proportions of soluble and insoluble true lignin fractions.

Digestibility trials showed that insoluble true lignin was not digested and could be used in the lignin ratio technique for calculating digestibilities indirectly. The soluble true lignin showed appreciable digestion, but the total amount of digestible lignin in feeding stuffs/

stuffs does not seem likely to exceed 1% so that lignin cannot be a significant source of energy for the animal.

The mathematical relation between the conventional and lignin ratio methods of determining digestibility revealed that for an accurate comparison of the true methods it is necessary to use a number of animals and an extended experimental period in order to diminish cumulative experimental errors.

Acknowledgement.

The writer wishes to thank Professor S. J. Watson and Dr. A. M. Smith for their interest in the subject and for their valuable guidance;

He also wishes to express his gratitude to Dr. F. E. Moon for his help, guidance and criticism and for his close supervision throughout the course of the investigation.

Thanks are also due to the members of the Chemistry Department of the Edinburgh and East of Scotland College of Agriculture who have helped from time to time.

The writer wishes to record his gratitude to the Egyptian Government whose financial support made this undertaking possible.

References.

Armitage, E.R. et al., J.S.C.I., 67: 241-43, 1948

Aronovsky, S.I.L., and Gortner, R.A., Ind.Eng.Chem., 22, 264, 1930.

Armstrong, D.G., et al., J. Agric. Sci., 40, 93-99 1950.

Association of Official Agricultural Chemists, Official and tentative methods of analysis, 6th Ed. A.O.A.C. Washington, D.C. 1945.

Ibid, 5th ed. 1940.

Bailey, Paper Ind. and Paper Word, 29, 1606-7, 1611, 1948 in C.A., 42, 2428b, 1948.

Bailey, A.J. Mikrochemie, 19, 98, 1936.

Bamford, K.F., and Campbell, W.G., Biochem. J. 30, 419 1939.6

Becker, E., Papier-Fabr., 17, 1325, 1919.

Beckman, E., et al. Z. angew, Chem., 34, 285, 1921.

Van Beckum W. and Ritter, G.J., Paper Trade J. 105, (18), T.S. 277, 1937.

Bell, A. and Wright, G.F., J.Amer.,Chem.Soc. 72:1495-99, 1950.

Bell, A., et al. J.Amer.Chem.Soc. 59: 598 1937.

Benedikt, R. and Bamberger, M., Monatsh., 11, 260, 1890.

Bennett, E., Soil Science, 68 : 399-400, 1949.

Bergeim, O., J. Biol.Chem., 70, 29, 1926.

Bondi, A., and Meyer, H., Biochem. J., 43, 248-56, 1948.

Bondi, A., and Meyer. H., J.Agric. Sci. 33 : 123-28, 1943.

Bondi, A., and Meyer, H. Bull. Rehovot Agric. Res. Sta. No. 27, 1940.

Boruff/

Boruff and Buswell., J. Amer.Chem.Soc., 56, 886,1934.

Brauns, F.E., J. Org. Chem., 10, 211, 1945.

Brauns, F.E., Paper Trade J.111, No.14, 33,1940.

Brauns, F.E., J.Amer.Chem.Soc.,61, 2120, 1939.

Brauns and Hibbert, J.Amer.Chem.Soc.,55, 4720, 1933.

Brauns, F. and Hibbert, H., Canad. J.Res. 13B, 78 1935.

Bray, M.W., Paper Trade J., 87, No.25:59,1928.

Breirem, K. Cellulose som for. (Cellulose for fodder), 1940. Papir Journalen, 28, 101-3; 113-5; 119-25; 133-36; 152-154, 1940.
in Nutri. Abs. 12: 146.

Brickman, L., et al. J.Amer.Chem.Soc., 62 : 2149,1940b

Brickman, L., et al. J.Amer.Chem.Soc., 62 : 986, 1940a

Brigl, P., and Pfahler, A., Tiererhahrung, 1, 30, 1929

Browne, C.A., J.Assoc.Off.Agric.Chem., 23, 102-108, 1940.

Browne, C.A., Dissertation, p.50, Gottingen,1901.

Bruel, D., et al., Biochem. Biophys. Acta, 1, 101, 1947.

Buchanan, M.A., et al., J.Amer.Chem.Soc., 71, 1297-9 1949.

Campbell, W.G. and McGowan, J.C., Nature, 143, 1022, 1939.

Campbell, W.G., et al., Biochem. J. 32, 2138, 1938.

Campbell, W.G., et al., Biochem. J. 31, 1285, 1937.

de Candole, A.P., "Pflanzen-Physiologie", Part 2, pp. 164-170. German translation by J. Ropper, Stuttgart and Tubingen, T.G., Cotta'schen, Buchhandung, 1835.

Carpenter, D.C., Analytical Chem. 20: 536, 1948.

Chastaignet/

Chastaignet, J., Me'm. services chim. état. (Paris) 34, 255-62, 1948. (in C.A. 44 : 7528h, 1950.)

Clark, E.P., J.Assoc., Official Agric. Chem. 15, 136-140, 1932.

Clark, E.P., Ind. Eng.Chem. Anal., ed.10, 677, 1938.

Cohen, W.E., and Harris, E.E., Ind.Eng.Chem.Anal. Ed. 9, 234, 1937.

Cohen, W.E., C.S.I.R., ~~1936,~~ Australia Pamphlet No.62, Div. Forest.Prods.,Tech. Paper, No.20.,1936.

Cohen, W.E. and Dadswell, H.E, C.S.I.R. Australia, Pamphlet No.22; Div. Forest Prods., Tech. Paper No.3, 1931.

Common, R.H., J.Agric.Sci., 35, 56-63, 1945.

Crampton, E.W., and Maynard, L.A., Jour.Nutri., 15, 383-395, 1938.

Crampton, E.W. and Whiting, F., J.Animal.Sci.,2, 278-84, 1943.

Crampton, J. "Advances in Carbohydrate Chemistry" 2 : 282, 1948.

Crocker, E.C., Ind.Eng.Chem., 13, 625, 1921.

Cross, C.F., and Bevan, E.J., "Researches on Cellulose" Vol. III, (1905-1910) p.104, London, Longmans,Green and Co., 1912.

Cross, C.F., et al., Chem. Ztg., 31, 725, 1907.

Csonka, F.A., et al., J.Biol.Chem., 85 : 65-75, 1929.

Davis, R.E. and Miller, C.O., Ind.Eng.Chem.Anal.Ed.11, 651-652, 1939.

Dietrich, T., and Konig, J., Landw. Vers.-Sta.,13, 222 1871.

Dore, W.H., Ind.Eng.Chem., 12, 476, 1920a.

Ibid 984, 1920b.

Dorée, C., "The Methods of Cellulose Chemistry", 2nd Ed. Chapman and Hall Ltd. 1947

Drapala/

Drapala et al., Sci.Agric., 27 : 37-41, 1947.

Druce, E., and Willcox, J.S. The Empire Journal of Experimental Agriculture, 17, 188-92, 1949.

Ducet, G. Ann.agron., 16, 219-25, 1946 (in C.A. 41, 4878e, 1947.)

Ellis, C.H., J. Assoc.Off.Agric.Chem. 32, 287, 1949.

Ellis, C.H. et al., J.Animal Sci., 5,285, 1946.

Erdtman, H. Research,(London) 3, 63-67, 1950.

Erdtman, H., Stvensk Papperstidn, 42, 115-22, 1939. (in C.A.33, 5385$_8$,1939.)

Von Euler, A.C., Cellulosechem. 4, 1, 1923.

Ferguson, W.S., Agric. Progress, 23, 129, 1948.

Ferguson, W.S., J.Agric.Sci. 33: 174-177, 1943.

Ferguson, W.S., Biochem. J. 36, 786-789, 1942.

Fernandez, O and Regueiro, B, Farm. nueva 11, 57-62, 1946. (in C.A., 5292h, 1947.)

Folin, O and Denis, W., J.Biol.Chem., 12, 239, 1912.

Forbes, E.B. et al., J.Animal. Sci., 5, 298-405,1946.

Forbes, R.M. and Garrigus, W.P., J.Animal Sci. 9, 551-59, 1950b.

Forbes, R.M. and Garrigus, W.P., J.Animal Sci. 9, 354-62,(1950a)

Forbes, R.M. and Garrigus,W.P., J.Animal Sci. 7, 373, 1948.

Forbes, R.M. and Garrigus, W.P., J.Animal Sci. 6, 493, 1947.

Freeman, R.D. "Wood Chemistry" Ed. Wise, L.E., Reinhold Publishing Corp. N.Y., 1946.

Fremy, E., Compt, rend, 48, 862, 1859.

Freudenberg/

Freudenberg, E., Sitzber. heidelberger Akad.wiss., Math-naturw. Klasse, 1949, No.5 151-8, 1949. in C.A. 44 : 2235d, 1950.

Freudenberg, K. and Richtzenhain, H. Holzforchung, 1, 90-4, 1947. in C.A. 44, 4671i, 1950.

Freudenberg, K. and Dloetz, T., Ber. 73B, 754-757, 1940.

Freudenberg, K., Ann.Rev. Biochem., 8, 88, 1939.

Freudenberg, K., J. Chem.Education, 9, 1171, 1932.

Freudenberg, K., et al. Cellulosechem. 12, 263, 1931.

Freudenberg, K., et al. Ber., 62, 1814, 1929b.

Freudenberg, K., et al. Ber. 62, 1554, 1929a.

Freudenberg, K. Sitzber, Heidelber. Akad.wiss. Math.Naturw. Klasse, Abbandlung, 19, 1928.

Friedrich, A., Z.physiol. Chem., 176, 127, 1928.

Fuchs, W., "Die Chemie des Lignins" J. Springer, Berlin, 1926.

Fuller, W.H., and Norman, A.G., J.Bact., 46, 291,1943.

Gallup, W.D. and Kuhlmann, A.H., J.Agric.Res. 52, 889, 1936.

Gallup, W.D. and Kuhlmann, A.H., J.Agric.Res. 42, 655, 1931

Glading, R.E. Paper Trade J. (Tappi) 111:288-295, 1940.

Goss, M.J. and Phillips, M.J., J.Assoc.Off.Agric.Chem. 19, 341, 1936.

Gottlieb, S. and Geller, J.H., Science 110, 189-190,1949. (See Goss and Phillips 1936)

Griffioen, K., Chem.Weekblad, 36, 81-5, 1939. (in C.A. 33: 6390$_8$, 1939.)

Gupta, A.B.S. Ph.D. Thesis, Leeds University, 1948. (cited by Druce & Willcox (1949))

Hachihama/

Hachihama, Y., and Jodai, S., J.Soc.Chem.Ind. Japan, 46, 132-9, 1943. (in C.A. 43, 2427g, 1949.)

Hagglund, E., Zellstoff, U. Papier 16: 570,1936. (in C.A., 31 : 2418, 1937.)

Hagglund, E. and Klingstedt, F.W. Z.Physik, Chem. 152, 295-312, 1931.

Hagglund, E. and Urban, H. Cellulosechem. 9, 49, 1928

Hagglund, E., and Urban, H. Cellulosechem. 8, 69, 1927.

Hagglund, E., and Bjorkman, C.B., Biochem. Z., 147, 74, 1924.

Hagglund, E., and Sundroos, B., Biochem. Z. 146, 221. 1924.

Hagglund, E., Ber., 56, 1866, 1923.

Hagglund, E., and Malm, C.J. Acta.Acad.Abensis, Math. Phys. 2. No.4, 1922.

Hale, E.B., et al., J.Nutrition, 34: 733-45, 1947.

Ibid 747-58, 1947.

Hale, E.B., et al., J. Dairy Sci., 23, 953, 1940.

Harlow, W.M. Bulletin of the New York State College of Forestry, 27, 6B,1927.

Ibid 1, 3, 1928.

Harris, E.E., and Mitchell, R.L. Ind.Eng.Chem.Anal. Ed.11 : 153, 1939.

Harris, E.E., et al., Biodynamica, No.31, pp.12,1937-8.

Hawk, P.B. et al., "Practical Physiological Chemistry" 12th Ed. Churchhill, J. and A.Ltd. London, 1949.

Heller, V.G., et al., J.Biol.Chem., 79, 275, 1928.

Henneberg, W. Beitrage zur Begrund-undungeine rationellen Futtering der Wiederkauer, Vol.1, 1860.

Heuser, E. and G.W.Wenzel, Papier Fabrk, 19, 1185, 1929.

Herzog/

Herzog, R.O., and Himler, A. Z.physiol.Chem. 168: 117-123, 1927. (in C.A., 21: 3204, 1927.)

Heuser, E. Cellulosechem., 4, 77, 84, 1923.

Heuser, E., J. prakt. Chem., 103, 69, 1921.

Heuser, E., and Schmelz, H., Cellulosechem., 1, 49, 1920.

Heuser, E., and Skioldebrand, C., Z.angew.Chem., 32, I, 41, 1919.

Hibbert, H. Ann.Rev.Biochem. 11, 183, 1942.

Hilpert, R.S. and Littmann, E., Ber. 68B, 16-18, 1935. (in C.A. 29: 2352$_7$, 1935.)

Hilpert, R.S., and Peres, O. Ber - 68B, 1575-8, 1935. (in C.A. 29: 7291$_7$, 1935.)

Hilpert, R.S., and Littmann, E., Ber. 67B, 1551-6, 1934. (in C.A. 29: 7251$_8$, 1934.)

Hilpert, R.S. and Wagner, R. , Ber. 68B, 571-80, 1935. (in C.A. 29: 3705$_8$, 1935.)

Hirst, E.L., and Jones, J.K.N., "Advances in Carbohydrate Chemistry" Vol.2. p. 235-51, Edit. Pigman, W.W.et al, Academic Press, Inc. Publishers, New York, N.Y., 1946.

Holmberg, B., Svensk Papperstidn, 39, Congress issue, 1936.

Jodl, R., Brennstoff. Chem. 20, 87-91, 128, 1939. (in C.A., 33, 9595$_9$, 1939.)

Kalb, L. Analyse des Lignins. In Klein, O., Handbuch der Pflanzenanalyse, 3, 1, 156-204, 1932.

Karrer, P., and Widmer, F., Helv.Chim.Acta., 4, 700, 1921.

Klason, P. Cellulosechem. 12, 37, 1931.

Klason, P., Cellulosechem. 4, 81, 1923.

Klason, P., Ber. 55, B, 448, 1922b.

Klason/

Klason, P., Paper Trade J. 74, No.18, 45,1922a

Klason, P., Ber. 53, B, 1864, 1920b.

Klason, P., Ber., 53, 706, 1862, 1864, 1920a

Klason, P., Arkiv.Kemi, Mineral,Geol.,3, No.5 17, 1908.

Knott, J.C., et al., J.Agric.Res., 53: 553-56, 1936.

Konig, J. and Becker, E., Z.angew, Chem.32, 1.155, 1919.

Konig, J., and Becker, E., Veroffentlichungen der Landwirtschaftskammer fur die Provinz Westfalen, Heft 26, 1918.

Konig, J. and Rump, E., Z. Nahr. Genussm,28,184,1914.

Konig, J., et al., Landw. Vers.-Sta., 65, 55, 1906.

Kormščikov, P.A., "Feeding of Farm Animals and Fodder Products" pp.254-261, Published by Lenin Acad. Agric. Sci., Moscow, 1940. in Nutri.Abs. 14, 2746: 566,1944.

Krull, H., Dissertation, Danzig, 1916.

Kukharenko, T.A., Doklady Acad. Nauk. S.S.S.R., 72, 907-10, 1950. (in C.A., 44, 818d, 1950.)

Kurschner, K. Cellulosechem. 18, 64-70, 1940. (in C.A., 34, 6440b, 1940.)

Kurshner, K., and Wittenberger, K. Papier fabr. 37, Tech.-wiss. Tl. 285-94, 297-303, 305-15, 1939. (in C.A. 33, 898b, 1939.)

Kurschner, K. "Zur Chemie der Ligninkorper", pp. 94-108. Stuttgart, F.Enke, 1925.

Lancaster, R.J. N.Z.J.Sci.Technol.(A), 25, 131-136: 137-151, 1943.

Lemmel, L. Anales Soc.espan, fis, quim. 33, 389-390, 1935.

Levine, M., et al. Ind.Eng.Chem., 27, 195, 1935.

Louw, J.G. Onderstepoort, J.Vet.Sci. and Animal Industry, 17, 165-179,1941.

Ma/

Ma, T.A., and Zuazaga, G., Ind.Eng.Chem.(anal.) 14, 280, 1942.

Manning, K.R. and DeLong, W.A., Sci.Agric., 22: 69, 1941.

Mattson, S., and Koulter-Andersson, E., Ann.Agr.Col. Sweden, 11: 107-134, 1943.

Maule, C., Beitrage wiss. Bot., 4, 166, 1900.

Mehta, M.M., Biochem. J. 19, 966, 1925b.

Mehta, M.M. Biochem. J. 19, 958, 1925a.

Moerke, G.A., J.Org.Chem. 10, 42-45, 1945.

Morrison, F.B., Feeds and Feeding, 20th ed. Morrison Publishing Co., Ithaca, N.Y. 1936.

Mueller, W.J. and Herrmann, W. Tech-Wiss.Teil, Papierfabr. 24: 185, 1926. (in C.A. 20: 2746, 1926.)

Müller, O.A. Papier-Fabr, 37, Tech.wiss.Tl. 184-188; ibid, Tl.221-226; ibid, Tl.237-40, 1939. (in C.A. 33, 8009, 1939.)

Müller, O., and Stoch, K. Ber. 72B, 73-76, 1939. (in C.A. 33, 2330, 1939.)

Muller, W.J. and Herrmann, W. Papier-Fabr, 24, 185, 1926. (See Mueller & Hermann, 1926)

McAnally, R.A. Biochem. J. 36, 392, 1942.

McCall, R., et al., Agr.Expt.Sta.Bull. 418, 30pp.1943; Expt.Sta.Record 91, 182, 1944. (in C.A. 40: 2556, 1946.)

McDougal, D., DeLong, W.A., Canad.J.Res., 26B6, 468-71, 1948c.

McDougal, D., DeLong, W.A., Canad.J.Res. 26B6: 464-67 1948b.

McDougal, D., DeLong, W.A., Canad.J.Res. 26B6, 457-63, 1948a.

McDougal, D., DeLong, W.A., Canad.J.Res. 20B, 40-48, 1942.

McKeekan, C.P. New Zealand J.Sci.Tech. 25 A, 152, 1943.

Naumann, K. Z. Tierernahr. Futtermittelk, 5, 193-246, 1940. (in C.A. 35: 1887, 1941.)

Nickel/

Nickel, E., Botan.Centr. 38, 755, 1889.
Noll, A., et al., Papier-Fabr., 32, 169, 1934; ibid, 30, 613, 1932.
Nord, F.F., and Vitucci, J.C., Adv.in Enxymol. 8, 253, 1948.

Norman, A.G., J.Amer.Soc.Agron. 31, 751, 1939.

Norman, A.G. "The Biochemistry of Cellulose the Polyuronides Lignin and C. Oxford at the Claredon Press, 1937.

Norman, A.G. and Richardson, P.L. Biochem.J. 31, 1556, 1937.

Norman, A.G. Biochem. J. 31: 1567, 1937.

Norman, A.G. Biochem. J. 30, 135, 1936.

Norman A.G. J.Agric. Sci, 25: 529-40, 1935.

Norman A.G. and Jenkins, S.H., Biochem.J. 28: 2160, 1934b.

Norman A.G. and Jenkins, S.H. Biochem.J. 28: 2147, 1934a.

Ogg, C.L. et al., J. Assoc.Off.Agr. Chem., 31, 663, 1948.

Overbeck, W., and Muller, H.F., Ber. 75B, 547-54, 1942 in C.A. 37: 761$_8$, 1943.

Paloheimo, L., Biochem. Z., 214, 161, 1929.

Paloheimo, L., Cellulosechem. 3, 19, 1928.

Paloheimo, L., Trans.Agr.Soc. Finland, Part 13, Helsinki, 1926.

Paloheimo, L., Biochem. Z., 165, 463, 1925.

Partansky, A.M. and Benson, H.K., Paper Trade J. 102, No.7, 29, 1936.

Patterson, R.F., and Hibbert, H., J.Amer.Chem.Soc. 65: 1869-1873, 1943.

Patton, A.K. and Cieseker, L., J.Animal Sci., 1, 22-26, 1942.

Payen, A., Compt. Rend. 9, 149, 1839.

Payen, A., Compt. Rend. 7, 1052, 1125, 1838.

Pazur/

Pazur, J.H. and DeLong, W.A., Sci.Agric. 28, 39-46, 1948.

Percival, E.G.V., and Fischer, H.E., Pulp Paper Mag. Can. 45, 17-23, 1944.

Perry, J.H., "Chemical Engineers' Handbook", McGraw-Hill Book Co., Inc. N.Y. and London, 1941.

Phillips, M. and Loughlin, T.C. J.Agric.Res., 78, 389, 1949

Phillips, M., in "Wood Chemistry" Edit. Wise, L.E., Reinhold Publishing Co., N.Y. 1946.

Phillips, M., J.Assoc.Off.Agri.Chem., 23, 108-19, 1940.

Phillips, M., J.Assoc.Off.Agric.Chem. 22, 422, 1939.

Phillips, M. et al., J.Agric. Res. 59, 319-366, 1939.

Phillips, M., J.Assoc.Off.Agric.Chem., 21, 678, 1938b.

Phillips, M., J.Assoc.Off.Agric.Chem., 21, 145, 1938a.

Phillips, M., and Goss, M.J., J.Assoc.Off.Agric.Chem. 21, 140, 1938.

Phillips, M., and Goss, M.J. J.Biol.Chem. 114, 557, 1936c

Phillips, M., and Goss M.J., J.Assoc.Off.Agric.Chem. 19, 350, 1936b.

Phillips M. and Goss, M.S., J.Assoc.Off.Agric.Chem. 19, 341, 1936a.

Phillips M., J.Assoc.Off.Agric.Chem., 18, 380, 1935.

Phillips M. and Goss M.J., J.Agric.Res., 51, 301, 1935.

Phillips, M., J.Assoc.Off.Agric.Chem., 15:118-31, 1932.

Phillips, M., Science, 73, 568, 1931.

Phillips, M., J.Amer.Chem.Soc. 52, 793, 1930.

Phillips/

Phillips, M., Proc.Soc.Exp.Biol.Med., 26, 320, 1929.

Phillips, M., et al., Proc.Soc.Exp.Biol., N.Y., 28, 320, 1928-29, 1929.

Phillips, M., J.Amer.Chem.Soc., 50, 1986, 1928.

Phillips, M., J.Amer.Chem.Soc., 49, 2037, 1927.

Poppoff, I.D., Z.Tierenahr. Frittermittelk, 1, 245, 1938.

Powell, W.J. and Whittaker, H., J.Chem.Soc., 127,132, 1925.

Pregl, F., "Quantitative Organic Chemistry" 4th english ed. J.Grant, J. and A. Churchhill Ltd., 1945.

Prjanischnikow, N.D., and Tomme, M.F., Biedermanns, Zentralbl. B. Tierenahrung, 8, 104-112, 1936.
in Nutr. Abs. 6, 1177:231, 1936.

Rathnow, H.D., Inaug. Diss., Munich, 1938.

Remy, E., Biochem. Z., 236, 1, 1931.

Ritter, G.J., et al., Ind.Eng.Chem.,anal.Ed. 4: 202-204, 1932.

Rogozinski, F. and Starzewska, M., Acta. Biol. Expt. Warsaw, 1, No.8, 1928.

Rogozinski, P. and Starzenwska, M., Intern.Rev.Agric; 18, 413, 1927.

Rogozinski, F. and Starzewska, M., Bull. intern.acad. Polonaise, Series B, 1-2,157,1926.

Ross, J.H., and Hill, A.C., Pulp and Paper Mag. Can. 27:241, 1929.

Roudier, A. Mém. services chim.état, 34, 263-72, 1948.
in C.A.44: 7528h, 1950.

Rubner, M., Die Naturwissenschaften, 16, 1011, 1928b

Rubner, M., Sitzber, preuss. Akad.Wiss.Physik. Math. Klasse, 12, 127, 1928a.

Russel/

Russel, A., J.Amer.Chem.Soc., 70:1060-64. 1948.

Rutledge, W.A., Common, R.H., J.Agric.Sci., 38, 28-32, 1948.

Rutledge, W.A., and Common, R.H., J.Agric.Sci., 37, 60963, 1947.

Sahyun, M. Ed., "Outline of Amino Acids and Proteins", Reinhold Publishing Co., 1944.

Schneider, B.H., "Feeds of the World", West Virginia, Agric.Expt.Sta. 1947.

Schorger, A.W., "Chemistry of Cellulose and Wood" p.524, London 1926.

Schubert, W.I. and Nord, F.F., J.Am.Chem.Soc. 72, 853-38, 1950a.

Schulbach, H.H. et al., Z.angew.Chem. 45, 245,1932.

Schultze, E., Z.physiol.Chem, 16, 387,1892.

Schulze, F., Chem. Zentr., 28, 321, 1857.

Schwalbe, C.G., and Ekenstam, A., Cellulosechem, 8, 13, 1927.

Schwalbe, H., Papier-Fabr., 23, 174, 1925.

Schwalbe, C.G., and Becker, E. Z.angew.Chem.32, I, 126, 1919.

Schwalbe, C.G., and Becker, E., Z.angew, Chem.32, I. 229, 1919.

Seidel, F., Disserrtation, Dresden, 1907. in Wise, L.E., "Wood Chemistry" p.635, 1946.

Semmler, F.W., and Pringsheim, H., Landw.Vers.Sta., 94, 85, 1919.

Sherrard, E.C., and Harris, E.E., Ind.Eng.Chem. 24, 103, 1932.

Siggia, S. "Quantitative Organic Analysis" John Wiley, N.Y. 1949.

Simmons, J.R. Paper Maker's Assoc.Gt.Brit. Ireland, Tech.Sect.Tech.Bull,17, 90-92, 1940.

Smith, A.M. Edin. and E. of Scotland College of Agric. Miscellaneous Publication No. 16, 1950.

Sowden, F.J. and DeLong, W.A., Sci.Agric. 29, 409-417 1949a

Ibid 418-423, 1949b.

Stamm, A.J. et al., J.Phys.Chem. 36: 1574-1584, 1932.

Standnikov, G.L.et al., Kolloid-Z.71, 206-214, 1935. (in C.A. 29: 7998 , 1935.)

Staudinger, H., et al., Ber. 69B, 1729, 1936.

Staudinger, H., and Dreher, E., Ber. 69, 1729, 1936.

Stutzer, A., Ber., 8, 575, 1875.

Swift, R.W., et al., J.Animal Sci., 6: 432-444, 1947.

Thomas, B. and Armstrong, D.G. J.Agric.Sci, 39, 335-346, 1949.

Thomas, B. and Ibbotson, C.F. J.Agric.Sci., 37, 58, 1947.

Trautmann, A., and Asher, T., Arch.viss, prakt. Tierheilk, 76, 317-328,1941. in Nutr.Abs. 11, 591, 1941-2.

Tweney, C.F. and Hughes, L.E.C., "Chamber's Technical Dictionary" revised edit. W.R. Chambers, Ltd., 1947.

Urban, H., Cellulosechem., 7, 73, 1926.

Venkateswaran, S., Quart.J.Indian Chem.Soc., 2, 253, 1925.

Virasoro, E., Anales inst. investigacionescient technol., 12/13, 175-186, 1943. (in C.A. 38, 6553 , 1944.)

Virasoro, E., Anal. Assoc. quim.argentina, 30, 54, 1942. (in C.A., 36, 6011, 1942.)

Waentig, P., and Gierisch, W. Z.angew.Chem., 32, I, 173, 1919.

Waentig, P., and Kerenyi, E., Papier Fabr. 18:920,1920.

Waksman/

Waksman, S.A., in "Wood Chemistry" Edit.Wise, L. E., Reinhold Publishing Corp.N.Y. 1946.

Waksman, S.A., and Smith, H.W., J.Amer.Chem.Soc., 56, 1225, 1934.

△Waksman, S.A., and Stevens, K.R., Ind.Eng.Chem.Anal. Ed.2, 167, 1930.

Watson, S.J., "Feeding of Livestock" 1st pub. Thomas Nelson and Son Ltd. 1949.

Watson, S.J., Brit.Soc.Anim.Prod. R., Rept. Proc. 9th meeting, Fe.18, 1948.

Watson, S.J., J.Roy.Agric.Soc.(England) 101, 37-41, 1941.

Wiechert, K., Papier-Fabr. 37, 325-32, 1939.

Ibid Ibid, 339-43, 1939 (in C.A. 34: 4901$_4$, 1940.)

Wiertelak, J., and Dzieragowski, M., Przemyl chem. 23, 227-234, 1939. (in C.A., 34, 3492$_8$, 1940.)

△Wenzl, H., Papier-Fabr. 22, 101, 1924.

△Williams, R.B. and Olmsted, W.H. J.Biol.Chem. 108, 653-66, 1935.

△Willstatter, R. and Kalb, L., Ber. 55, 2637, 1922.

Wise, L.E. Chemist Analyst 36, 8, 1947. (in C.A., 5055$_i$, 1947)

Wise, L.E. et al., Paper Trade J. 122, 2,35, 1946.

△Wise, L.E. and Fairbrother, A.M., Ind.Eng.Chem.Anal, Ed. 3, 253, 1931.

Woodman, H.E., "Rations for Live-stock" British Min.Agric.Fish. Bull.No.48,G.B. 1948.

Woodman, H.E., Evans, R.E., J.Agric.Sci. 37:202-210, 1947a.; ibid, 37:211-223, 1947b.

Woodman, H.E. and Stewart, J., J.Agric.Sci., 22, 527-547. 1932.

△ These references are cited by Phillips (1946).